

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau



38
BY

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁵ : C12N 05/10, 15/63, 15/82 C07H 15/12, A01H 5/00	A1	(11) International Publication Number: WO 92/17580 (43) International Publication Date: 15 October 1992 (15.10.92)
(21) International Application Number: PCT/US92/02822 (22) International Filing Date: 7 April 1992 (07.04.92) (30) Priority data: 682,354 8 April 1991 (08.04.91) US (60) Parent Application or Grant (63) Related by Continuation US 682,354 (CON) Filed on 8 April 1991 (08.04.91) (71) Applicant (for all designated States except US): RHONE-POULENC AGROCHIMIE [FR/FR]; 14-20, rue Pierre-Baizet, F-69263 Lyon (FR).	(72) Inventors; and (75) Inventors/Applicants (for US only) : THOMAS, Terry [US/US]; 3004 Normand, College Station, TX 77845 (US). FREYSSINET, Georges [FR/FR]; 21, rue de Nervieux, F-69450 Saint-Cyr-au-Mont-d'Or (FR). LEBRUN, Michel [FR/FR]; 224, rue de Saint-Cyr, F-69009 Lyon (FR). BOGUE, Molly [US/FR]; 20A, rue des Glacières, F-67000 Strasbourg (FR). (74) Agents: SCOTT, Anthony, C. et al.; Scully, Scott, Murphy & Presser, 400 Garden City Plaza, Garden City, NY 11530 (US). (81) Designated States: AT (European patent), AU, BE (European patent), BR, CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, KR, LU (European patent), MC (European patent), NL (European patent), SE (European patent), US. Published <i>With international search report.</i>	
(54) Title: CHIMERIC PLANT GENES BASED ON UPSTREAM REGULATORY ELEMENTS OF HELIANTHININ (57) Abstract Helianthinin is an 11S seed storage protein of sunflower embryos. The present invention is directed to the 5' regulatory regions of helianthinin genes. More particularly, the present invention is directed to specific cis-regulatory elements of this regulatory region which direct tissue-specific, temporally-regulated, or abscisic acid-responsive gene expression. The present invention provides chimeric genes comprising the cis-regulatory elements linked to a coding sequence from a heterologous gene to control expression of these genes. The chimeric genes provided by the instant invention are useful in conferring herbicide resistance and improved seed lipid quality to transgenic plants.		

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	ES	Spain	MG	Madagascar
AU	Australia	FI	Finland	ML	Mali
BB	Barbados	FR	France	MN	Mongolia
BE	Belgium	GA	Gabon	MR	Mauritania
BF	Burkina Faso	GB	United Kingdom	MW	Malawi
BG	Bulgaria	GN	Guinea	NL	Netherlands
BJ	Benin	GR	Greece	NO	Norway
BR	Brazil	HU	Hungary	PL	Poland
CA	Canada	IT	Italy	RO	Romania
CF	Central African Republic	JP	Japan	RU	Russian Federation
CG	Congo	KP	Democratic People's Republic of Korea	SD	Sudan
CH	Switzerland	KR	Republic of Korea	SE	Sweden
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
DE	Germany	MC	Monaco	TG	Togo
DK	Denmark			US	United States of America

CHIMERIC PLANT GENES BASED ON UPSTREAM
REGULATORY ELEMENTS OF HELIANTHININ

1

Helianthinin is an 11S seed storage protein of sunflower embryos. The present invention is directed to the 5' regulatory regions of helianthinin genes. More particularly, the present invention is directed to specific cis-regulatory elements of this regulatory region which direct tissue-specific, temporally-regulated, or abscisic acid-responsive gene expression. The present invention provides chimeric genes comprising the cis-regulatory elements linked to a coding sequence from a heterologous gene to control expression of these genes. The chimeric genes provided by the instant invention are useful in conferring herbicide resistance and improved seed lipid quality to transgenic plants.

15

Seed development, unique to higher plants, involves embryo development as well as physiological adaptation processes that occur within the seed to ensure the survival of the developing seedling upon germination. After fertilization, there is rapid growth and differentiation of the embryo and endosperm, after which nutritive reserves accumulate during the maturation stage of seed development. These reserves are stored during a period of developmental arrest for later use by the developing seedling. This period of arrest occurs prior to the desiccation phase of seed development.

20

Several classes of seed proteins, including storage proteins, lectins, and trypsin inhibitors, accumulate during embryogenesis. The main function of seed storage proteins is to accumulate during embryogenesis and to store carbon and nitrogen reserves for the developing seedling upon germination. These proteins, as well as many of the genes encoding them, have been studied extensively (for review see Shotwell et al. (1989) in The Biochemistry of Plants, 15, Academic Press, NY, 297).

25

30

35

1 Genes encoding seed storage proteins are highly
regulated and differentially expressed during seed development.
Expression is temporally regulated with mRNA accumulating
rapidly during the maturation phase of embryogenesis. This
5 expression is also tissue-specific, occurring primarily in the
cotyledons or endosperm of the developing seeds. The resulting
storage proteins are processed and targeted to protein bodies,
in which the storage proteins remain during desiccation and
dormancy of the embryo. Upon germination, the seedling uses
10 these storage proteins as a source of carbon and nitrogen
(Higgins (1984) Ann. Rev. Plant Physiol. 35, 191).

Seed proteins, including storage proteins, lectins and
trypsin inhibitors, are encoded by nonhomologous multigene
families that are not amplified or structurally altered during
15 development (for review see Goldberg et al. (1989) Cell 56,
149). These genes are temporally and spatially regulated but
not necessarily linked. Although post-transcriptional
mechanisms act to control the accumulation of some of these
proteins, regulation occurs primarily at the transcriptional
20 level. Accordingly, seed protein genes provide an excellent
system to provide genetic regulatory elements, especially those
elements which confer tissue specificity, temporal regulation,
and responsiveness to environmental and chemical cues.

Observations of temporal and spatial regulation of
25 seed protein genes has suggested that seed protein genes are
regulated in part by common cellular factors known as trans-
acting factors. However, since quantitative and qualitative
differences exist in the expression patterns of individual seed
protein genes, more specific factors must also exist to provide
30 a means for differential expression patterns between these
groups of seed proteins. Patterns of differential expression
have been observed between the rapeseed major seed storage
proteins, cruciferin and napin (Crouch et al. (1981) Planta 153,

-3-

1 64; Finkelstein et al. (1985) Plant Physiol. 78, 630), and among
individual members of the soybean Kunitz trypsin inhibitor gene
family (Jofuku et al. (1989) Plant Cell 1, 1079). A comparison
5 of the soybean major seed storage protein genes showed a
difference in timing and cell-type specificity of the expression
of β -conglycinin (7S) and glycinin (11S). The 7S subunit mRNA
appeared several days before the 11S mRNA. Furthermore, while
members of the glycinin gene family were all activated
simultaneously (Nielsen et al. (1989) Plant Cell 1, 313),
10 members of the β -conglycinin gene family were differentially
regulated (Barker et al. (1988) Proc. Natl. Acad. Sci. USA 85,
458; Chen et al. (1989) Dev. Genet. 10, 112). Each of these
genes contain a different array of cis-regulatory elements which
confer differential expression patterns between, and within,
15 these gene families.

Helianthinin is the major 11S globulin seed storage
protein of sunflower (Helianthus annuus). Helianthinin
expression, like that of other seed storage proteins, is tissue-
specific and under developmental control. However, the
20 helianthin regulatory elements which confer such specificity
have heretofore never been identified. Helianthinin mRNA is
first detected in embryos 7 days post flowering (DPF) with
maximum levels of mRNA reached at 12-15 DPF, after which the
level of helianthinin transcripts begins to decline. In mature
25 seeds or in germinating seedlings helianthinin transcripts are
absent. Helianthinin polypeptide accumulation is rapid from 7
DPF through 19 DPF but slows as the seed reaches later
maturation stages (Allen et al. (1985) Plant Mol. Biol. 5, 165).

Helianthinin, like most seed proteins, is encoded by
30 a small gene family. At least two divergent subfamilies are
known, and are designated Ha2 and Ha10. Two clones, HaG3-A and
HaG3-D, representing non-allelic members of the Ha2 subfamily,
have been isolated and partially characterized (Vonder Haar et

1 al. (1988) Gene 74, 433). However, a detailed analysis of the
regulatory elements of these or any other helianthinin genes had
not been known until now.

5 It has been found in accordance with the present
invention that regulatory elements from helianthinin genes can
direct seed-specific gene expression, root-specific gene
expression, abscisic acid-responsive gene expression, and/or
temporally-altered gene expression. These regulatory elements
10 enable the controlled expression of specific gene products in
transgenic plants. The present invention provides greater
control of gene expression in transgenic plants, thus allowing
improved seed quality, improved tolerance to environmental
conditions such as drought, and better control of herbicide
resistance genes.

15 The present invention is directed to the 5' regulatory
region of a helianthinin gene. This region is herein referred
to as the upstream regulatory ensemble (URE), and is useful in
directing the expression of heterologous proteins. The URE
consists of multiple regulatory elements which confer distinct
20 regulated expression patterns when linked to the coding regions
of heterologous genes which are expressed in transgenic plants.

In particular, the present invention provides isolated
DNA containing helianthinin regulatory elements which direct
seed-specific gene expression, root-specific gene expression,
25 abscisic acid (ABA)-responsive gene expression and/or
temporally-altered gene expression.

Another aspect of this invention is directed to
chimeric plant genes containing these regulatory elements. The
regulatory elements are operably linked to the coding sequence
of a heterologous gene such that the regulatory element is
30 capable of controlling expression of the product encoded by the
heterologous gene. If necessary, additional promoter elements
or parts of these elements are included in the chimeric gene

-5-

1 constructs. Plant transformation vectors comprising the
chimeric genes of the present invention are also provided, as
are plant cells transformed by these vectors, and plants and
their progeny containing the chimeric genes.

5 In yet another aspect of this invention, a method is
provided for producing a plant with improved seed-lipid quality.
Chimeric genes are constructed according to the present
invention in which a regulatory element directing seed-
specific expression is linked to the coding region of a gene
10 encoding a lipid metabolism enzyme. When plant cells are
transformed with this chimeric gene, plants with improved seed
lipid-quality can be regenerated.

A further aspect of the present invention provides a
method for producing a herbicide-resistant plant. In accordance
15 with the present invention, for example, chimeric genes are
constructed in which a root-specific regulatory element directs
the expression of herbicide-resistance gene. Plant cells are
transformed with this chimeric gene to regenerate herbicide-
resistant plants.

20 Fig. 1 depicts the nucleotide sequence of the URE of
helianthinin gene HaG3-A. Nucleotide numbers -2377 to +24 of
Fig. 1 correspond to nucleotide numbers 1 to 2401 of SEQ ID
NO:1.

25 Fig. 2 depicts the nucleotide sequence of part of the
URE of helianthinin gene HaG3-D. Nucleotide numbers -2457 to -
726 of Fig. 2 correspond to nucleotide numbers 1 to 1732 of SEQ
ID NO:2.

30 Fig. 3 represents the nucleotide sequence of part of
the URE of helianthinin gene HaG3-D. Nucleotide numbers -725
to -322 of Fig. 3 correspond to nucleotide numbers 1 to 404 of
SEQ ID NO:3. In the helianthinin gene HaG3-D, the nucleotide
sequence of Fig. 3 is immediately downstream (3') of the
sequence of Fig. 2.

35

1 Fig. 4 depicts the HaG3-A FL/GUS construction and the control constructions pBI121.1 and pBI101.1.

5 Fig. 5 depicts a restriction map of helianthinin genomic clones HaG3-A and HaG3-D and the restriction fragments used to construct the parental plasmids.

 Fig. 6 depicts the HaG3-A and HaG3-D derivative constructions in relation to the full length construction.

10 Fig. 7 demonstrates histochemical localization of GUS activity in transgenic seedlings containing the HaG3-D-N and HaG3-A-SB/R constructions. A: HaG3-D-404N, 8 days post-imbibition (DPI); B: HaG3-A-SB/R, 8 DPI; C: HaG3-D-404N, 14 DPI; D: HaG3-A-SB/R, 14 DPI; E: HaG3-A-SB/R, 8 DPI; F: HaG3-A-SB/R, 6 DPI.

15 Fig. 8 graphically illustrates the induction of GUS activity in transgenic tobacco leaves containing HaG3-D-404N during progressive desiccation and subsequent recovery from water deficit.

 Fig. 9 is a graph depicting ABA induction of GUS expression in leaves of tobacco containing HaG3-D-404N.

20 The present invention comprises cis-regulatory elements of the upstream regulatory ensemble (URE) of sunflower helianthinin genes. These cis-regulatory elements are discrete regions of the URE that confer regulated expression upon the gene under their control. In particular, this invention provides isolated nucleic acid containing at least one regulatory element from a helianthinin gene which directs at least one of the following: seed-specific gene expression, root-specific gene expression, ABA-responsive gene expression or temporally-altered gene expression. Any helianthinin gene can provide the regulatory elements, including Ha2 and Ha10 genes, which represent two divergent helianthinin gene subfamilies. In a preferred embodiment, the helianthinin genes are HaG3-A and HaG3-D, which are members of the Ha2 subfamily.

35

-7-

1 One of the subject regulatory elements directs seed-
specific expression. A seed-specific regulatory element
represents a particular nucleotide sequence that is capable of
causing the expression of the gene under its control to occur
5 in the seed, i.e. for the gene produced to be detected in the
seed. Expression that is seed-specific may be in any part of
the seed, e.g., but not limited to, the cotyledons and embryonic
axis of the embryo and to the endosperm. No gene expression is
detected in seedlings or somatic tissues of the adult plant for
10 genes under seed-specific control.

To identify regulatory elements that direct seed-
specific expression, a deletion analysis of the entire URE of
a helianthinin gene can be performed. In a deletion analysis,
nucleotides are successively removed from the entire URE, and
15 the resulting fragments are ligated to the coding sequence of
a reporter gene or other heterologous gene. The constructs are
then analyzed for their ability to direct seed-specific
expression by detecting the presence of the reporter gene
product in seed tissues and not in other tissues. The seed-
20 specific elements which have been identified can also be
modified, e.g. by site-directed mutagenesis. The modified
regulatory elements can then be assayed for their ability to
direct seed-specific expression, thereby identifying alternative
sequences that confer seed-specificity. These techniques for
25 identifying regulatory elements are applicable to all
helianthinin genes. For example, in a preferred embodiment an
analysis of the URE of the helianthinin HaG3-A gene indicates
that seed-specific regulatory elements are provided by
nucleotides 851 to 2401, and by nucleotides 1 to 2401 of SEQ ID
30 NO:1.

Other regulatory elements provided by the present
invention provide root-specific expression. Root-specific
expression is of particular interest and importance. Normally

35

1 the sunflower helianthinin gene is expressed only in seeds.
When particular regions of the helianthinin URE are isolated
from the entire URE in accordance with the present invention,
expression is exclusively localized to plant roots. A root-
5 specific regulatory element represents a particular nucleotide
sequence that is capable of causing the expression of the gene
under its control to occur in plant roots and not in other plant
tissues. Regulatory elements that direct root-specific
expression are identified by analyzing fragments of a
10 helianthinin URE for their ability to confer root-specific
expression as described above for the identification of seed-
specific regulatory elements except expression is detected in
root tissues. Modifications of the nucleotide sequences that
permit root-specific expression are also identified as described
15 above. Root-specific regulatory elements from any helianthinin
gene can be identified by such techniques. For example, in a
preferred embodiment, an analysis of the URE of the helianthinin
HaG3-A gene indicates that nucleotides 1 to 1639 and nucleotides
851 to 1639 of SEQ ID NO:1 represent root-specific regulatory
20 elements.

Helianthinin expression is under strict temporal
control, with mRNA first detected at 12 DPF. Accordingly, it
has been discovered that cis-regulatory elements exist which
confer temporally-altered gene expression which is detectable
as early as about 4 DPF.

25 To identify regulatory elements that confer
temporally-altered gene expression, a deletion analysis of the
entire URE of a helianthinin gene can be performed. Fragments
of the URE are linked to the coding sequence of a heterologous
gene and the resulting chimeric construction is used to
30 transform plants. Seeds from transformed plants are staged by
days post flowering, and the staged seeds are assayed to detect
the expression of the heterologous gene. Elements that direct

-9-

1 expression of the heterologous gene before about 10 DPF are
identified as elements that confer temporally-altered
expression. Modifications of the nucleotide sequences of such
elements that confer the desired phenotype can be identified as
5 described above. These techniques for identification of
regulatory elements that confer temporally-altered gene
expression are applicable to all helianthinin genes. In a
preferred embodiment, an analysis of the URE of the helianthinin
gene HaG3-A indicates that elements that confer temporally-
10 altered gene expression are provided by nucleotide 1 to 851 and
1639 to 2303 of SEQ ID NO:1.

Another aspect of the present invention is directed
to regions of the URE of helianthinin that confer abscisic acid
(ABA)-responsive gene expression. An ABA-responsive element
15 represents a particular nucleotide sequence that is capable of
causing the gene under its control to be expressed in response
to ABA. Expression of the gene under the control of the ABA-
responsive element can be induced by treatment with ABA, or by
external stimuli that are known to result in the initiation of
20 ABA biosynthesis. For example, ABA biosynthesis is initiated
as a result of loss of turgor caused by environmental stresses
including water-deficit, water-stress and salt-stress (reviewed
in Zeevaart et al. (1988) Annu. Rev. Plant Physiol. 39, 439).
Levels of ABA also increase in response to wounding, (Peña-
25 Cortes et al. (1989) Proc. Natl. Acad Sci. USA 86, 9851).
ABA-responsive elements are identified as described above for
the identification of other regulatory elements. For example,
deletion analysis can be used to identify nucleotide sequences
of any helianthinin gene that induce the expression of a gene
30 under its control in response to ABA. Such sequences can be
modified as described above, and assayed to identify alternative
sequences that confer ABA-responsive expression. In one
preferred embodiment, an analysis of the URE of the helianthinin

35

1 HaG3-A gene indicates that nucleotides 1 to 2401 of SEQ ID NO:1
provide an element that confers ABA-responsive expression in
seeds. In another preferred embodiment, nucleotides 851 to 1639
or 1639 to 2303 of SEQ ID NO:1 provide an element that confers
5 ABA-responsive expression in leaves of adult plants. In yet
another preferred embodiment, an analysis of the URE of the
helianthinin HaG3-D gene indicates that nucleotides 1 to 404 of
SEQ ID NO:3 confer ABA-responsive expression in non-embryonic
tissues of plants.

10 Accordingly, ABA-responsive elements have utility in
that specific environmental cues can initiate ABA biosynthesis,
and further induce expression of genes under the control of an
ABA-responsive element. Expression of heterologous genes driven
by the ABA-responsive elements of the helianthinin URE is not
15 restricted to seeds, but is also observed in leaves of adult
plants and in tissues of seedlings.

An isolated nucleic acid encoding the upstream
regulatory ensemble of a helianthinin gene can be provided as
follows. Helianthinin recombinant genomic clones are isolated
20 by screening a sunflower genomic DNA library with a cDNA
recombinant representing helianthinin mRNA (Vonder Haar (1988)
Gene 74, 433). Methods considered useful in obtaining
helianthinin genomic recombinant DNA are contained in Sambrook
et al., 1989, in Molecular Cloning: A Laboratory Manual, Cold
25 Spring Harbor, NY, for example, or any of the myriad of
laboratory manuals on recombinant-DNA technology that are widely
available. To determine nucleotide sequences, a multitude of
techniques are available and known to the ordinarily skilled
artisan. For example, restriction fragments containing a
30 helianthinin URE can be subcloned into the polylinker site of
a sequencing vector such as pBluescript (Stratagene). These
pBluescript subclones can then be sequenced by the double-strand
dideoxy method (Chen and Seeburg (1985) DNA 4, 165).

-11-

1 The nucleotide sequence for DNA encoding the URE of
helianthinin gene clone HaG3A is shown in Fig. 1 and presented
as SEQ ID NO:1. Similarly, the nucleotide sequence for DNA
5 encoding a region of the URE of helianthinin clone HaG3D is
shown in Fig. 2 and presented as nucleotide sequence SEQ ID
NO:2. The UREs of other helianthinin genes can be obtained by
the same strategy. Alternatively, clones representative of
other members of the helianthinin gene family can be obtained
by using the HaG3A or HaG3D coding or URE sequences of the
10 present invention as hybridization probes to screen a
helianthinin genomic library and identify the additional
helianthinin genes.

 The identification of cis-regulatory sequences that
direct temporal, tissue-specific and ABA-responsive regulation
15 can be accomplished by transcriptional fusions of specific
sequences with the coding sequence of a heterologous gene,
transfer of the chimeric gene into an appropriate host, and
detection of the expression of the heterologous gene. The assay
used to detect expression depends upon the nature of the
heterologous sequence. For example, reporter genes, exemplified
20 by chloramphenicol acetyl transferase and β -glucuronidase (GUS),
are commonly used to assess transcriptional and translational
competence of chimeric constructions. Standard assays are
available to sensitively detect the reporter enzyme in a
transgenic organism. The β -glucuronidase (GUS) gene is useful
25 as a reporter of promoter activity in transgenic tobacco plants
because of the high stability of the enzyme in tobacco cells,
the lack of intrinsic β -glucuronidase activity in higher plants
and availability of a qualitative fluorimetric assay and a
histochemical localization technique. Jefferson *et al.* [(1987)
30 EMBO J, 6, 3901]] have established standard procedures for
biochemical and histochemical detection of GUS activity in plant
tissues. Biochemical assays are performed by mixing plant

-12-

1 tissue lysates with 4-methylumbelliferyl- β -D-glucuronide, a
fluorimetric substrate for GUS, incubating one hour at 37°C, and
then measuring the fluorescence of the resulting 4-methyl-
5 umbelliferone. Histochemical localization for GUS activity is
determined by incubating plant tissue samples in 5-bromo-4-
chloro-3-indolyl-glucuronide (X-Gluc) for 18 hours at 37°C, and
observing the staining pattern of X-Gluc. The construction of
such chimeric genes allows definition of specific regulatory
sequences required for regulation of expression, and
10 demonstrates that these sequences can direct expression of
heterologous genes in the manner under analysis.

Another aspect of the present invention is directed
to a chimeric plant gene containing a regulatory element from
a helianthinin gene which directs seed-specific gene expression,
15 root-specific gene expression, ABA-responsive gene expression
or temporally-altered gene expression linked to the coding
sequence of a heterologous gene such that the regulatory element
is capable of controlling expression of the product encoded by
the heterologous gene. The heterologous gene can be any gene
20 other than helianthinin. If necessary, additional promoter
elements or parts of these elements sufficient to cause
expression resulting in production of an effective amount of the
polypeptide encoded by the heterologous gene are included in the
chimeric constructs.

25 Accordingly, the present invention provides chimeric
genes comprising regions of the helianthinin URE that confer
seed-specific expression in accordance with this invention which
are linked to a sequence encoding a lipid metabolism enzyme such
as a desaturase. In a preferred embodiment, the regions of the
30 URE comprise nucleotides 851 to 2401 or 1 to 2401 of HaG3-A as
shown in SEQ ID NO:1. Any modification of these sequences which
confers seed-specific expression is contemplated. Seeds
accumulate and store proteins and lipids, both of significant

35

-13-

1 agronomic importance. Because elements of the helianthinin URE
can direct high, regulated expression in developing seeds, these
elements have utility in improving seed lipid and/or protein
quality. These elements are useful in regulating expression of
5 genes encoding lipid metabolism enzymes, such as those involved
in elongation and desaturation of fatty acids, and/or proteins,
especially those with high lysine and methionine content.
Chimeric genes containing these elements can be used to provide
transgenic plant lines that accumulate and store significant
10 amounts of specific classes of lipids and/or proteins.

In another aspect of the present invention chimeric
genes are provided which have a region of the URE of
helianthinin that confers root-specific expression fused to a
heterologous gene. This construction confers expression
15 spatially distinct from "normal" helianthinin expression in that
the heterologous gene is expressed exclusively in plant roots.
In other words, when a specific sequence is removed from the
context of the entire URE, tissue-specific regulation is
altered. In a preferred embodiment, the region of the HaG3-A
20 URE comprises 1 to 1639 or 851 to 1639 of SEQ ID NO:1 and is
fused in reverse orientation to the promoter although these
elements function in either orientation. In another preferred
embodiment the sequence providing herbicide resistance is at
least part of the aroA gene. Any modification of these
25 sequences which confers root-specific expression is
contemplated.

Of particular importance is the use of these chimeric
constructions to confer herbicide resistance. Since most
herbicides do not distinguish between weeds and crop plants, the
30 engineering of herbicide-resistant crop plants is of
considerable agronomic importance in that it allows the use of
broad-spectrum herbicides. Accordingly, the present invention
provides chimeric genes comprising elements of a helianthinin

35

-14-

1 URE that confer root-specific expression fused to at least part
of a promoter that functions in plants and further fused to at
least part of the *aroA* gene or a sequence encoding a polypeptide
conferring herbicide resistance. Polypeptides that confer
5 resistance to glyphosate and related inhibitors of 5-
enolpyruvylshikimic acid-3-phosphate synthase (EPSP synthase),
sulfonyleureas, imidazolinones and inhibitors of acetolactase
synthase (ALS) and acetohydroxy acid synthase (AHS) are
contemplated. In a preferred embodiment the regions of the URE
10 are 1 to 1639 or 851 to 1639 of HaG3-A, as shown in SEQ ID NO:1
and are fused in reverse orientation to the promoter. Any
modification of these sequences which confers root-specific
expression is contemplated.

15 In another aspect of the present invention chimeric
genes are provided comprising elements of the URE of
helianthinin that confer temporally-altered expression fused in
forward or reverse orientation to at least part of a promoter
that functions in plants and further linked to the coding region
of a heterologous gene. In a preferred embodiment the elements
20

25

30

35

-15-

1 of the URE are nucleotides 1 to 851 or 1639 to 2303 of HaG3-A,
as shown in SEQ ID NO:1. Any modification of these sequences
that confers temporally altered gene expression is contemplated.

5 Chimeric genes are provided comprising elements of the
URE of a helianthinin that confer ABA-responsive expression
optionally fused in forward or reverse orientation to at least
part of a promoter that functions in plants further fused to a
heterologous gene. In a preferred embodiment the element of the
10 URE comprises 851 to 1639 or 1639 to 2303 of HaG3-A, as shown
in SEQ ID NO:1, or nucleotides 1 to 404 of HaG3-D, as shown in
SEQ ID NO:3. Of particular importance is the use of constructs
that confer ABA-responsive expression to provide plants with
improved tolerance to water stress.

15 The chimeric genes of the present invention are
constructed by fusing a 5' flanking sequence of a helianthinin
genomic DNA to the coding sequence of a heterologous gene. The
juxtaposition of these sequences can be accomplished in a
variety of ways. In a preferred embodiment the order of
sequences, from 5' to 3', is a helianthinin upstream regulatory
20 region, a promoter region, a coding sequence, and a
polyadenylation site.

Standard techniques for construction of such chimeric
genes are well known to those of ordinary skill in the art and
can be found in references such as Sambrook *et al.* (1989). A
25 variety of strategies are available for ligating fragments of
DNA, the choice of which depends on the nature of the termini
of the DNA fragments. One of ordinary skill in the art
recognizes that in order for the heterologous gene to be
expressed, the construction requires promoter elements and
signals for efficient polyadenylation of the transcript.
30 Accordingly, the 5' helianthinin URE regions that contain the
promoter sequences known as CAAT and TATA boxes can be fused
directly to a promoterless heterologous coding sequence.

35

1 Alternatively, the helianthinin URE regions that do not contain
the CAAT and TATA boxes can be joined to a DNA fragment encoding
a promoter that functions in plants. Plant promoters can be
obtained commercially, or can be chemically synthesized based
5 on their published sequences. An example of such a fragment is
the truncated cauliflower mosaic virus 35S promoter, which
retains its CAAT and TATA boxes. Other representative promoters
include the nopaline synthase and ribulose 1,5 biphosphate
carboxylase promoters. The promoter fragment is further linked
10 to the heterologous coding sequence. The 3' end of the coding
sequence is fused to a polyadenylation site exemplified by, but
not limited to, the nopaline synthase polyadenylation site.
Furthermore, intermediate plant transformation vectors are
available that contain one or more of these polyadenylation
15 sites bordered by sequences required for plant transformation.
The elements of the helianthinin URE and the heterologous coding
sequences of the present invention can be subcloned into the
polylinker site of a plant transformation vector to provide the
chimeric genes.

20 The 5' flanking elements of the present invention can
be derived from restriction endonuclease or exonuclease
digestion of a helianthinin genomic clone. The restriction
fragments that contain the helianthinin CAAT and TATA boxes are
ligated in a forward orientation to a promoterless heterologous
25 gene such as the coding sequence of β -glucuronidase (GUS). The
skilled artisan will recognize that the 5' helianthinin
regulatory sequences can be provided by other means, for example
chemical or enzymatic synthesis. The heterologous product can
be the coding sequence of any gene that can be expressed in such
30 a construction. Such embodiments are contemplated by the
present invention. The 3' end of the coding sequence is
optionally fused to a polyadenylation site, exemplified by, but
not limited to, the nopaline synthase polyadenylation site, or

-17-

1 the octopine T-DNA gene 7 polyadenylation site. Alternatively,
the polyadenylation site can be provided by the heterologous
gene.

5 The 5' helianthinin regulatory elements that do not
contain the TATA box can be linked in forward or reverse
orientation to at least part of a plant promoter sequence, i.e.
a plant promoter sequence containing at least the CAAT and TATA
sequences. In a preferred embodiment, this promoter is a
truncated cauliflower mosaic virus (CaMV) 35S promoter. The
10 resulting chimeric complex can be ligated to a heterologous
coding sequence and a polyadenylation sequence.

To provide regulated expression of the heterologous
genes, plants are transformed with the chimeric gene
constructions of this invention. Gene transfer is well known
15 in the art as a method to express heterologous genes in
transgenic plants. The tobacco plant is most commonly used as
a host because it is easily regenerated, yields a large number
of developing seeds per plant, and can be transformed at a high
frequency with Agrobacterium-derived Ti plasmid vectors (Klee,
et al. (1987) Annu. Rev. Plant Physiol. 38, 467).
20 Dicotyledenous plants including cotton, oil seed rape and
soybean are preferred as transgenic hosts. However, one of
ordinary skill in the art will recognize that any plant that can
be effectively transformed and regenerated can be used as a
transgenic host in the present invention.

25 A variety of transformation methods are known. The
chimeric genes can be introduced into plants by a leaf disk
transformation-regeneration procedure as described by Horsch et
al. (1985) Science 227, 1229). Other methods of transformation,
such as protoplast culture (Horsch et al., (1984) Science 223,
30 496; DeBlock et al. (1984) EMBO J. 2, 2143; Barton et al. (1983)
Cell 32, 1033) or transformation of stem or root explants in
vitro (Zambryski et al. (1983) EMBO J. 2, 2143; Barton et al.

35

1 (1983) Cell 32, 1033) can also be used and are within the scope
of this invention. In a preferred embodiment plants are
transformed with Agrobacterium-derived vectors. However, other
5 methods are available to insert the chimeric genes of the
present invention into plant cells. Such alternative methods
include biolistic approaches (Klein et al. (1987) Nature 327,
70) electroporation, chemically-induced DNA uptake, and use of
viruses or pollen as vectors.

10 When necessary for the transformation method, the
chimeric genes of the present invention can be inserted into a
plant transformation vector, e.g. the binary vector described
by Bevan (1984). Plant transformation vectors can be derived
by modifying the natural gene transfer system of Agrobacterium
15 tumefaciens. The natural system comprises large Ti (tumor-
inducing)-plasmids containing a large segment, known as T-DNA,
which is transferred to transformed plants. Another segment of
the Ti plasmid, the vir region, is responsible for T-DNA
transfer. The T-DNA region is bordered by terminal repeats.
In the modified binary vectors the tumor-inducing genes have
20 been deleted and the functions of the vir region are utilized
to transfer foreign DNA bordered by the T-DNA border sequences.
The T-region also contains a selectable marker for antibiotic
resistance, and a multiple cloning site for inserting sequences
for transfer. Such engineered strains are known as "disarmed"
25 A. tumefaciens strains, and allow the efficient transformation
of sequences bordered by the T-region into the nuclear genomes
of plants.

30 Surface-sterilized leaf disks are inoculated with the
"disarmed" foreign DNA-containing A. tumefaciens, cultured for
two days, and then transferred to antibiotic-containing medium.
Transformed shoots are selected after rooting in medium
containing the appropriate antibiotic, and transferred to soil.

-19-

1 Transgenic plants are self-pollinated and seeds from these plants are collected and grown on antibiotic-containing medium.

Expression of a heterologous or reporter gene in developing seeds, young seedlings and mature plants can be
5 monitored by immunological, histochemical or activity assays.

As discussed herein, the choice of an assay for expression of the chimeric gene depends upon the nature of the heterologous coding region. For example, Northern analysis can be used to assess transcription if appropriate nucleotide probes
10 are available. If antibodies to the polypeptide encoded by the heterologous gene are available, Western analysis and immunohistochemical localization can be used to assess the production and localization of the polypeptide. Depending upon the heterologous gene, appropriate biochemical assays can be
15 used. For example, acetyltransferases are detected by measuring acetylation of a standard substrate. The expression of an herbicide-resistance gene can be detected by determining the herbicide resistance of the transgenic plant.

Another aspect of the present invention provides
20 transgenic plants or progeny of these plants containing the chimeric genes of the invention. Both monocotyledenous and dicotyledenous plants are contemplated. Plant cells are transformed with the chimeric genes by any of the plant transformation methods described above. The transformed plant
25 cell, usually in a callus culture or leaf disk, is regenerated into a complete transgenic plant by methods well-known to one of ordinary skill in the art (e.g. Horsch et al. (1985) Science 227, 1129). In a preferred embodiment, the transgenic plant is cotton, oil seed rape, maize, tobacco, or soybean. Since
30 progeny of transformed plants inherit the chimeric genes, seeds or cuttings from transformed plants are used to maintain the transgenic plant line.

1 The instant invention also provides a method for
producing a plant with improved seed lipid quality. This method
comprises transforming a plant cell with a vector containing a
chimeric gene comprising a seed-specific regulatory element
5 linked to the coding sequence of a lipid metabolism enzyme such
as a desaturase, and selecting for a plant with the desired
characteristics. In a preferred embodiment the regulatory
element is provided by nucleotides 1 to 2401 or 851 to 2401 of
the URE of HaG3A as shown in SEQ ID NO:1. The transformed plant
10 cells are regenerated into plants with improved seed lipid
quality.

 Another aspect of the present invention provides a
method for producing a plant with improved seed protein quality.
This method comprises transforming a plant cell with a vector
15 containing a chimeric gene comprising a seed-specific regulatory
element linked to the coding sequence of a seed storage protein
with a high content of lysine and/or methionine residues, and
selecting for a plant with the desired characteristic. In a
preferred embodiment the regulatory element is provided by
20 nucleotides 1 to 2401 or 851 to 2401 of the URE of HaG3-A as
shown in SEQ ID NO:1. The transformed plant cells are
regenerated into plants with improved seed protein quality.

 Another aspect of the present invention provides a
method for producing a herbicide-resistant plant. Plant cells
25 are transformed with a vector containing a chimeric gene
comprising a root-specific regulatory element linked to the
coding sequence of a herbicide resistance gene such as a
glyphosate resistance gene and then plants with the desired
herbicide resistance are selected. Selected plants are those
30 which survive a herbicide treatment which kills untransformed
plants of the same kind under the same conditions. In a
preferred embodiment, the regulatory element is provided by
nucleotides 1 to 1639 or 851 to 1639 of the URE of HaG3-A as

-21-

1 shown in SEQ ID NO:1, and the heterologous sequence is provided
by a gene encoding EPSP synthase, acetolactase synthase, or
acetohydroxy acid synthase. The transformed plant cells are
5 regenerated into herbicide-resistant plants. In a preferred
embodiment, plants are transformed by the vector pRPA-ML-803,
which contains the root-specific regulatory element comprising
nucleotides 851 to 1639 of HaG3-A and the aroA herbicide-
resistance gene.

10 The following examples further illustrate the
invention.

15

20

25

30

35

EXAMPLE 1General Methods

The nucleotide sequences referred to in the following examples are numbered according to Fig. 1-3.

GUS Reporter Gene Constructions

The general purpose GUS reporter cassettes used throughout the examples have been described previously (Jefferson et al. (1987) EMBO J. 6, 3901). Briefly, the coding region of GUS was ligated 5' of the nopaline synthase polyadenylation site in the polylinker site of the A. tumefaciens-derived vector pBIN19 (Bevan (1984) Nucleic Acids Res. 12, 8711). The vector pBIN19 contains the left and right borders of T-DNA necessary for plant transformation, and a kanamycin resistance gene. The resulting construction, pBI101.1, is depicted in Figure 4. Unique restriction sites upstream of the AUG initiation codon of GUS allow the insertion of promoter DNA fragments.

The CaMV 35S promoter was ligated into the HindIII and BamHI sites of pBI101.1 to create pBI121.1, depicted in Fig. 4. To create pBI120, the CaMV 35S promoter was truncated at an EcoRV site at -90 (leaving the CAAT and TATA boxes) and cloned into the polylinker site of pBI101.1.

Table 1 describes the parental plasmids and derivative constructions. HaG3-A-FL and the control constructions pBI121.1 and pBI101.1 are depicted in Figure 4. Figure 5 shows the restriction fragments of genomic clones HaG3-A and HaG3-D used to construct parental plasmids. Figure 6 shows the derivative constructions schematically in relation to the full length construction.

The HaG3-A/GUS constructions represent large overlapping fragments that span the full length regulatory region (-2377 to +24 of Fig. 1). The 3' ends of several

-23-

1 constructions were derived from exonuclease III digestions of
a 2.8 kb HaG3-A fragment in pBluescript (Stratagene) [pHaG3-A-
2.8 (BamHI-PstI), Table 1]. These deletions are shown at the
top of Figure 4. The first deletion, pHaG3-A-2.4, contains the
5 HaG3-A CAAT and TATA boxes with its 3' end at -75. Fragments
that contained the HaG3-A CAAT and TATA boxes were ligated in
forward orientation into the promoterless GUS cassette pBI101.1.
Fragments that did not contain the HaG3-A TATA box were ligated
in both orientations upstream of the truncated CaMV 35S promoter
10 of pBI120. These fragments were subcloned into the appropriate
GUS cassette. Constructions are named according to their end
sites followed by an F, indicating forward orientation; R,
indicating reverse orientation. Arrows indicate the orientation
of the fragment with respect to the GUS coding region (Fig. 4).
15 The HaG3-D/GUS constructions contain a 404 bp fragment (SalI-
HpaI) in both orientations: Normal (N) and Inverse (I). The
accuracy and orientation of each construction was confirmed by
double-stranded dideoxy sequencing (Chen and Seeburg, 1985)
using primers to regions in the GUS cassettes (Advanced DNA
20 Technologies Lab, Texas A&M University).

Plant Transformation

The BIN-19 based plasmid constructions were used to
transform tobacco (Nicotiana tabacum cv. Xanthi) according to
standard procedures (Horsch et al. 1985) except that initial
25 transformants were selected on 50 µg kanamycin/ml and then were
transferred to 100 µg/ml kanamycin. Plants were self-
pollinated, and seeds were germinated on kanamycin (400 g/ml)
to identify transformants, since the BIN-19 based constructions
contain the neomycin phosphotransferase gene (NPTII), which
30 confers resistance to the toxic antibiotic kanamycin. The copy
number of each GUS construction integrated into the tobacco
genome was estimated for each transformant by segregation
frequencies of the NPTII gene. Most of the transformants

35

-24-

1 contained only one segregating locus of the construction.
Filial, homozygous plants were used where indicated. Transgenic
plants representing all of the test constructions were obtained
except for the reverse construction of H- 2. Transgenic plants
5 were maintained in Conviron chambers: 16h light:8h dark, 24°C,
70-80% relative humidity. All plants were watered on a strict
schedule to prevent desiccation prior to testing.

10

15

20

25

30

35

-25-

TABLE 1

<u>Construction</u>		<u>Description</u>
<u>Parental Plasmids</u>		
5	pBI101.1	Bin 19-derived promoterless GUS reporter gene cassette.
	pBI121.1	CaMV 35S promoter fused to GUS cassette in pBI 101.1.
10	pBI120	CaMV 35S promoter truncated at EcoRV site, leaving CAAT and TATA boxes, fused to GUS coding region.
	pHaG3-A-2.8	2.8 kb BamIH-PstI fragment of HaG3-A in pBluescript; contains 2.4 kb upstream of HaG3-A coding region and 0.38 kb downstream of transcription start site; used to generate exonuclease III deletions.
15	pHaG3-A-2.4	2.4 kb HaG3-A fragment generated from 3' exonuclease III digestion of pHaG3A-2.8 to +24; contains the HaG3-A CAAT and TATA boxes.
20	pHaG3-A-2.3	2.3 kb HaG3-A fragment generated from 3' exonuclease III digestion of pHaG3A-2.8 to -75; contains the HaG3-A CAAT box.

25

30

35

1 Derivative Constructions

- HaG3-A-FL 2.4 kb insert of pHaG3A-2.4 fused to pBI101.1 in forward orientation
- 5 HaG3-A-HS/F 0.85 kb BamH1-SalI fragment from pHaG3A-2.3
-HS/R cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter of pBI120.
- HAG3-A-HS/R 0.85 kb excised as a SacI fragment from HaG3A-HS/F and cloned in reverse orientation with respect to the truncated CaMV 35S promoter pBI 120.
- 10 HaG3-A-HB/F 1.6 kb BamH1-BalI fragment from pHaG3A-2.3
-HB/R cloned in forward and reverse orientations with respect to the truncated CaMV 35S promoter pBI 120.
- 15 HaG3-A-S 2/F 0.6 kb SalI-BalI from HaG3-A cloned in forward
-S 2/R and reverse orientations with respect to the truncated CaMV 35S promoter of pBI120;
constructed by deleting SalI-BamH1 fragment from HaG3-A-HB/F and HaG3-A-HB/R, respectively.
- HaG3-A-S 2/F 1.4 kb SalI fragment from pHaG3-A-2.3 cloned in
-S 2/R forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.
- 20 HaG3-A-B 2/F 0.66 kb BalI SalI fragment from pHaG3-A-2.3
-B 2/R cloned in forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.
- 25 HaG3-A-H 2 2.3 kb insert from pHaG3-A-2.3 cloned in forward
orientation with respect to the truncated CaMV 35S promoter of pBI120.
- HaG3-A-S 1 1.5 kb SalI fragment from pHaG3-A-2.4 cloned
in forward orientation with respect to pBI 101.1.
- 30 HaG3-D-404N 0.4 kb SalI-HpaI fragment form HaG3-D cloned in
-404I forward and reverse orientation with respect to the truncated CaMV 35S promoter in pBI120.

-27-

EXAMPLE 2

Biochemical Detection of GUS Activity:
Seed-Specific and Root-Specific Expression

GUS activity was determined in embryonic and non-embryonic tissues of transgenic tobacco containing each construction of Table 1. The standard procedures of Jefferson et al. (1987) were followed.

Plant tissue was ground in extraction buffer (50 mM NaPO₄, 10 mM EDTA, 0.1% Sarkosyl, 0.1% Triton X-100 and 10 mM β -mercaptoethanol). After centrifugation of the lysate, the supernatant was removed to a fresh tube and dispensed in 100 μ l aliquots. An equal volume of 2 mM 4-methylumbelliferyl- β -D-glucuronide in extraction buffer was added and allowed to incubate at 37°C for 1 h. Reactions were stopped with 0.8 ml Na₂CO₃ (0.2 M). The fluorescence of the resulting 4-methylumbelliferone (4-MU) was determined with a Hoeffler TKO-100 minifluorometer as described (Jefferson et al. 1987). GUS activity is expressed in picomoles 4-MU per unit mass total protein sample per minute.

Cotyledons, hypocotyls, leaves, and roots from transgenic seedlings, ranging from 18 to 20 days post-inhibition (DPI), containing various sequence elements of HaG3-A (summarized in Fig. 4) driving GUS expression were assayed for activity. Results are provided in Table 2. All constructions containing some portion of the URE of the helianthinin genes HaG3-A and HaG3-D conferred GUS activity in transgenic tobacco seeds. The full length regulatory region (FL) and fragments derived from this region, as well as the HaG3-D/GUS constructions, all conferred significant GUS activity in mature seeds when compared with the GUS expression driven by the intact CaMV 35S promoter complex (pBI121). However, well-defined seed-specific expression was only obtained with constructs including

1 the proximal upstream regions between -75 and +24 (cf. FL and
S- 1). These two constructions containing nucleotides -2377 to
+24 or -1527 to +24 demonstrated tissue-specific GUS expression
5 with no detectable GUS activity in any tissues of transgenic
seedlings. The FL construct, however, was expressed in mature
seeds at sixfold higher levels compared to S- 1. GUS activity
in tissues of seedlings containing the intact CaMV 35S promoter
complex (pBI121) are included for comparison as well as the
10 negative controls containing the truncated CaMV 35S promoter
(pBI120) or no promoter (pBI101). Compared to expression in
seeds there was little expression in leaves containing the same
construction; on the other hand, most constructions, other than
FL and S- 1, demonstrated significant expression in roots of
transgenic seedlings.

15 The overall activity conferred by the intact CaMV 35S
promoter complex was higher than that conferred by all other
constructions in somatic tissue except in roots. In particular,
roots of seedlings containing the HB/R (-2377 to -739) and SB/R
(-1527 to -739) constructions showed levels of GUS activity 7
20 to 8 times above that of roots expressing GUS under control of
the intact CaMV 35S promoter.

25

30

35

1

TABLE 2

Summary of GUS Expression in Embryonic and
Non-Embryonic Tissues of Transgenic Tobacco"

5

CONSTRUCTION ¹⁾		DEVELOPMENTAL PROFILE ²⁾	GUS ACTIVITY (pmole 4MU/μg/min) ³⁾			ABA Response ⁴⁾
			SEEDS ⁵⁾	LEAF ⁶⁾	ROOT ⁷⁾	
<u>HaG3-A</u>						
10	FL	I	18.7±8.7	0	0	+
	S- 1	I	3.4±1.1	0	0	ND
	F	III	17.1±15	0.45±0.05	36.6±2.2	-
15	HS	R	ND ^{a)}	0.23±0.05	8.9±1.8	-
	F	II	14.8±5.2	0.95±0.13	29.9±2.2	+
	HB	ND	13.1±6.9	0.25±0.05	75.4±3	-
20	F	II	11.1±5.8	0	13.9±6.8	-
	SB	ND	12.1±5.8	0.34±0.05	90.5±9.9	+
	F	II	35.7±4.2	0	20.6±10.2	ND
25	S- 2	ND	21.0±15	0.45±0.08	38.8±1.2	+
	F	III	11.2±3.9	2.03±0.08	8.0±0.62	+
	B- 2	ND	7.2±2.3	4.05±0.10	3.9±0.3	+
30	H- 2 F	III	1.8±1.0	ND	1.8±0.3	ND
	<u>HaG3-D</u>					
	N	III	9.2±2.9	0.07±0.01	6.8±0.5	+
35	404	I	9.2±3.9	2.03±0.05	12.9±2.8	+
	<u>Controls</u>					
	pBI 101	ND	0	0	0	-
35	pBI 120	ND	0	0	0	-
	pBI 121	ND	4.3±1.0	22.0±7.9	9.9±4.0	-

TABLE 2. (continued)

- 1 a Mature seeds and seedling tissues of transgenic tobacco containing constructions in Fig. 1 were assayed for GUS activity.
- 5 b Constructions are as shown in Fig. 1. Forward (F) and Reverse (R), Normal (N) and Inverted (I), refer to the orientation of each helianthinin fragment with respect to the truncated 35S CaMV promoter.
- 10 c Developing seeds of transgenic tobacco containing forward constructions in Fig. 1 were assayed for GUS activity at approximately 2 days intervals from 8-24 DPF. Type I, II and III profiles are defined in Example 3.
- d ND, not determined in this experimental series.
- e In all experiments, GUS assays represent averages from four to ten independently transformed plants for each construction. Standard deviations are included.
- 15 f GUS activity in mature (30 DPF) transgenic tobacco seeds.
- g Transgenic tobacco seedlings were grown axenically on solid medium. Tissues from seedlings (18-20 DPI) were collected and assayed for GUS activity.
- 20 h FL ABA responsive only in developing seeds 12-18 DPF (see text and Table 3). All others, ABA response predicted from GUS expression of dessicated leaves and subsequent demonstration that seedlings of indicated plants respond directly to exogenous ABA. Plus sign indicates induction of GUS activity over basal level. Minus sign indicates no detectable induction of GUS activity.

-31-

EXAMPLE 3Biochemical Detection of GUS Activity:
Temporally-Regulated Expression

1
5 The temporal profile conferred by each forward construction was determined and the results are shown in Table 2. Filial homozygous plants were grown and allowed to flower, and seeds from staged pods were assayed for GUS expression as described in Example 2. Three types of developmental profiles were identified based on the time of initial appearance of GUS activity in developing embryos and the qualitative and quantitative characteristics of the resulting expression patterns; Type I profiles showed correct temporal regulation where accumulation of GUS begins 12 DPF. In plants exhibiting Type II profiles, GUS activity also began accumulating around 12 DPF but peaked around 14 DPF followed by significant declines in levels of GUS activity. Type III plants showed activity occurring before 10 DPF with a peak of activity occurring at approximately 12 DPF. Constructions containing the regions of the HaG3-A URE from nucleotides -2377 to -1527 or -739 to -75 conferred this temporally earlier profile.

10
15
20
25
30
35

EXAMPLE 4Histochemical Localization of GUS Activity

1
5 GUS activity was histochemically localized in seedlings containing HaG3-A-SB/R and HaG3-D-404N. Samples were washed in 50 mM NaPO₄ and incubated for 24 h at 37°C in 100µl reaction buffer [50 mM NaPO₄, pH 7.0, 2 mM 5-bromo-4-chloro-3-indolyl-glucuronide (X-Gluc), 0.1 mM potassium ferricyanide, and 0.1 mM potassium ferrocyanide]. Samples were mounted on microscope slides with 80% glycerol.

10 HaG3-D-404N (Fig. 7A) and HaG3-A-SB/R (Fig. 7B) seedlings grown on basal media containing 1% sucrose showed slightly different patterns of expression. HaG3-D-N driven GUS expression appeared at low levels in the cotyledons and at significantly higher levels in the distal root region with no detectable activity in the hypocotyl. The HaG3-A-SB/R seedling also showed significant GUS activity in the distal root with no detectable activity in the hypocotyl or cotyledons. GUS activity was histochemically localized at 14 DPI in seedlings containing HaG3-D-404N that were grown in a water-deficient environment on sub-saturated filter paper; GUS activity was primarily in the leaves and roots of these seedlings (Fig. 7C).

15 The GUS expression patterns of seedlings containing HaG3-A-SB/R was determined. The major site of GUS activity in the SB/R seedling was in the developing root tips (Fig. 7B, C). In 6 DPI seedlings containing HaG3-A-SB/R, GUS was expressed throughout the length of the elongating root with particularly high levels in the meristematic region of the root tip (Fig. 7D). Histochemical localization of HaG3-A-SB/R seedlings (14 DPI) showed activity in newly formed lateral roots as well as the continued activity in the meristematic region of the main root (Fig. 7B). Seedlings from 16 DPI continued to show this

-33-

1 pattern of expression (Fig. 7C); root hairs and the distal portions of the root had high levels of GUS activity as well.

5

10

15

20

25

30

35

EXAMPLE 5ABA-Responsive Expression

1
5 In a series of whole plant experiments on transgenic tobacco containing constructions illustrated in Fig. 4, several regions of the UREs of HaG3-A and HaG3-D were identified that responded to changes in the plants water potential (Table 2). Since ABA is a known mediator of water-deficit responses, the effect of ABA on GUS expression driven by these elements was
10 determined. Within HaG3-A, two regions (-1527 to -739 and -739 to -75) were shown to confer ABA-responsive expression in leaves of mature transgenic tobacco and in seedlings. Another ABA-responsive element was identified in the URE of HaG3-D (-739 to -322).

15 The induction of GUS activity in transgenic tobacco containing HaG3-D-404N (forward orientation) was correlated with water potential during processive desiccation and subsequent recovery from water deficit. Since the full length HaG3-A URE is not expressed under any conditions except during seed
20 development, plants containing this chimeric GUS construction were used as negative controls. Filial, homozygous plants containing each construction were grown in soil. Plants were either watered normally (control) or stressed to varying degrees by watering with 1/3 the amount of the control plant or by not
25 watering at all. Fully stressed plants containing HaG3-D-404N were induced rapidly with a peak of GUS activity at about 36 hours, which correlated with a decrease in water potential (Fig. 8). Subsequent GUS determinations 24 hours later revealed a reproducible decrease in GUS activity even though the plants
30 were under severe water-deficit with water potential of nearly -4 bars. The fully stressed plants were recovered by watering after sampling was completed on day 3. The plants recovered quickly as the water potential returned to non-stressed levels

-35-

1 after watering, and GUS activity continued to decrease over the
remaining days. GUS activity in 1/3 stressed plants containing
HaG3-D-N increased more moderately during a 3.5 day interval as
5 the water potential decreased (Fig. 8). As observed with fully
stressed plants, GUS activity decreased before water-deficit
recovery. In no instance did the FL plants express GUS in non-
embryonic tissues.

To determine if the 404 bp fragment from HaG3-D
responds directly to ABA, leaf disks of transgenic tobacco
10 containing HaG3-D-404N were treated with ABA for increasing
periods of time and were subsequently assayed for GUS
expression. After a lag-time of approximately 3.5 hours,
treatment with 10 mM ABA resulted in a rapid increase in GUS
expression; GUS continued to accumulate through eight hours at
15 which time the rate of accumulation decreased significantly
(Fig. 9). There was no detectable GUS activity in leaf disks
from the same plant maintained under identical conditions
exclusive of ABA. Likewise, leaf disks from plants containing
the HaG3-A full length URE showed no activity during the course
20 of the experiment. Since the chimeric gene including the
CaMV35S promoter and the β -glucuronidase reporter gene is
transcriptionally active in leaves (Table 1), transgenic plants
containing pBI121 served as an important negative control. Leaf
disks from plants containing pBI121 showed no increase in GUS
25 activity in response to exogenous ABA throughout the experiment
(+ABA: 12.6 ± 3.3 pmole 4-MU/ μ g/min; -ABA: 13.5 ± 3.6 pmole 4-
MU/ μ g/min).

A similar series of experiments was carried out with
transgenic tobacco seedlings containing HaG3-D-404N and HaG3-A-
30 FL (Fig. 4). Eighteen DPI seedlings were transferred to media
containing 0-10 mM ABA, and GUS activity was determined one, two
and three days later (Table 3). Seedlings containing HaG3-D-
404N were inducible by ABA by day 1 at all ABA concentrations;

1 there was no significant induction of HaG3-A-FL in parallel
experiments. Induction was concentration and time dependent.
Maximum induction, exceeding 200 fold, occurred at two and three
days at ABA concentrations of 10 mM (Table 3). Significant
5 induction of 19 and 70 fold occurred on day three at 0.1 mM and
1.0 mM ABA, respectively.

The full-length (FL) helianthinin HaG3-A URE (-2377
to +24) was tested for its inducibility by ABA in developing
seeds. Seeds containing the full length (FL) regulatory region
10 driving the expression of GUS (Fig. 4) were staged at 11, 14,
18 and 24 days post flowering and were tested for their ability
to respond to ABA. Induction by ABA was shown by the increased
levels of GUS activity over levels obtained on basal media;
results are summarized in Table 3. ABA responsiveness varied
15 with the stage of development. Seeds from 11 DPF did not
respond to ABA during the course of the experiment whereas more
mature seeds did respond. Seeds from 14 DPF responded rapidly
with induction above basal levels beginning as early as 1.5
hours. There was a monotonic increase in GUS activity with 14
20 DPF seeds treated with ABA; by three days of treatment, the
levels of GUS activity were higher than that for 18 and 24 DPF
seeds treated with or without ABA. Seeds from 18 DPF were
slower to respond to ABA than those from 14 DPF, but levels of
GUS activity comparable to 14 DPF (+ABA) seeds were observed in
25 18 DPF seeds by the fifth day of ABA treatment. Seeds from 24
DPF are less responsive to ABA through five days of ABA
treatment. Levels of GUS activity also varied with seeds
incubated on basal media alone. Seeds from 14 DPF on basal
media continued to increase in GUS activity an estimated 4 pmol
30 4-MU/seed/day.

The preceeding results demonstrate a hierarchy
controlling helianthinin gene expression so that the ABA-
responsive elements contained within the HaG3 UREs are

-37-

1 functional only within the context of the appropriate
developmental program, i.e. seed maturation. Taking the ABA-
responsive elements out of the context of the HaG3-A or HaG3-D
5 UREs results in the loss of hierarchical control so that these
elements are free to respond directly to ABA and indirectly to
desiccation in leaves and seedlings of transgenic tobacco.

10

15

20

25

30

35

TABLE 3
ABA Induction In Vitro of HaG3-A-FL in
in Transgenic Tobacco Seeds"

GUS Activity (pmole 4-MU/μg/min)									
DPF	Normal	11 DPF		14 DPF		18 DPF		24 DPF	
ABA									
		+	-	+	-	+	-	+	-
11	0	0	0	--	--	--	--	--	--
14	7±0.3	0	0	7.0	7.0	--	--	--	--
16	--	0	0	--	--	--	--	--	--
17	--	--	--	33	15	--	--	--	--
18	15±0.3	--	--	--	--	15	15	--	--
19	--	--	--	57	24	--	--	--	--
21	--	--	--	--	--	24	15	--	--
23	--	--	--	--	--	61	15	--	--
24	16±2.0	--	--	--	--	--	--	16	16
27	--	--	--	--	--	--	--	21	16
29	--	--	--	--	--	--	--	24	16
35	14±2.0	--	--	--	--	--	--	--	--

a Transgenic tobacco seeds containing HaG3-A-FL were collected at indicated days post flowering (DPF) and were incubated on basal media alone or basal media containing 1 μ M ABA. GUS activity was determined after 0, 3 and 5 days of treatment. In vivo expression of HaG3-A-FL-driven GUS in developing seeds (Normal) is shown for reference.

1

5

15

20

30

35

-40-

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Thomas, Terry
Freyssinet, Georges
Lebrun, Michel
Bogue, Molly
- (ii) TITLE OF INVENTION: Chimeric Plant Genes Based on Upstream
Regulatory Elements of Helianthinin
- (iii) NUMBER OF SEQUENCES: 3
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Scully, Scott, Murphy & Presser
 - (B) STREET: 400 Garden City Plaza
 - (C) CITY: Garden City
 - (D) STATE: New York
 - (E) COUNTRY: USA
 - (F) ZIP: 11530
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: McNulty, William E.
 - (B) REGISTRATION NUMBER: 22,606
 - (C) REFERENCE/DOCKET NUMBER: 8081
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 516-742-4343
 - (B) TELEFAX: 516-742-4366

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2401 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

-41-

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GGATCCTCTA CCTATATATA TATATATATA TGAATTTTTT AAAAAAATCC CGTACCCCTC	60
GAAAAACGG GCCTTATGCG GAAGTCCTCC TCGCACACCT AAAGAGCCGC CCATGCTTTT	120
TAATCAAATA GATGTGCATC ATGTAGTGAT AGTTTTTACT AAAATCCATT AGTTTATAAA	180
TATTTTAAAT GTTTTTTTTT GTTTATATAA AAAAAGAAAA TTAAAAACA AAATGTCCAA	240
AATACTCCTG TATCAACTAT GCAAAAAGAC AAAAAACCC TTTTGGTTAA CAAAGTCTTT	300
AATTTAACTA AGTTTGTCAT TTGAAGGAAA TTCAAACAAA AACGAACGTG GGGGCGCGGG	360
GGTGGGGTGT TTGGTTACAA AAAGTTTAA TTTTAGATTA AAGTATAAAA ATTGCCCAAA	420
CCTCAGGACA ATTTTACAT TTATAACTCA TTGTCTAAAT ACTAAAATAC ACCAAGTCAA	480
TGGGTGAAAG TTACTATCTT TTTTATTGCA ATTTACATT ACCTTATTTA CTTTTGAGAA	540
AGACGACATA ACAATTAAGG AGTTATAGTC TGATCGGTTT GCGCTATTTT TCATACTTAA	600
GGTCCAGGTT TGAATCTTTT AAACATTTTT TTTTAACTTG ATCATAACAA TATAACAATT	660
AAGGAGTTAT GATCTGATGG TTTGCGTTAT GTTTTCGTAC TAATTAAGGT CCCGGTTTGA	720
ATCTCTCAAA CAATATATTA TTTTTCTTA AAAACGAATG AGACATGCTC ACAATGGGAA	780
TTGAACCGAC ACCTATTGGT TTAAAATTAA AGCTATAACA AACTGAGCTA CACATTTTTA	840
ATTTAAAAAT GTCGACTATC TTAGTTAATC AAATAAATTT ATTTTGATTT GTTTTGTTAA	900
TGTATTTTCT CCTAATTAA AGTCGATGTG TATTTATATA ATATTAGTAA TATTTATTA	960
ACATCAATAC ATGCTTCAGG TTTTGTGTTA GTCTTCGTTT TTTATATGGT TTTATCAGTG	1020
GTGTGGTGTA CGATGACGAT TATTTAAATA ATGACGAACT TCTTGGTTGT TACTTATTGA	1080
TGTACGAAGC TGAGATGTAA CGAACCGAAC ACATATAAAT AACATTTTGG ATAAGATTAC	1140
GACTTTATTT ATCGGTTGCC ATGAAATTTA GAAGATTTGG GTTAAGACAC AACCACATAT	1200
AATGTGATGG TAAATAGCAT TTACAATAA TGTTAATCTT TTGTTACAAA TGTTGTTAAC	1260
TAGGCTTGAT ATGTAAAATT TTTAAAGACT ATCAGGTGTT CTTACGGTTT TACATCTAGT	1320
AAGAGATTAA AAAAAAAAAA GCAAGGAAAG TAAGTGTAAG GAGAGTAAAG AGAATGTAGC	1380
CATGATATGG CTGATTGTTT ATCACCATCC CATTTATACT TATCATCTTG ATGATGCATA	1440
TAGACATGAT GTGTGCTACG TACCGAATTT TAACAGCTTC CCGGCGCAAC ACACGTGTAT	1500
AAATACCATA GATTATAAAC CAAATACGCT ACGTATAGGT GGTTATATGA TACCTATGAT	1560
GACTTGACCT TTCGTTACAC TTGAGCTGAA AAAAATAAAA AAATGTGGCT ATAGGCGCAT	1620

GGTCACAGTT TTTTGTGTG GCCATATACA ATTTTGTGACG TAGCGTTAGT TAATCAGATA 1680
 AATTTATTTT GATTTGTTTT GTTAATGTAT TTTCTCCTAA TTTCAAGTAG ACGTGTATTT 1740
 ATATAATATT AGTAATATTT TATTAACATC AATACATGCT TCATGTTTTG GGTAGTCTT 1800
 CGTTTTTTAT ATGGTTTTAT CAGTGGTGTA CGATGACGAT TATTTAAATA ATGACGGACT 1860
 TCTTGGTTGT TACTTATTGA TGTACGAAGC TGAGATGTAA CGAACCGAAC ACATATAAAT 1920
 AACATTTTGG ATAAGATTAC GACTTTATTT ATCGGTTGCC ATGAAATTG GAAGACTTGG 1980
 GTTAAGACAC AACCACATAT AATGTGATGG TAAATAGCAT TTACAATAA TGTTAATCTT 2040
 TTGTTACAAA TGTTGTTAAC TAGGCTTGAT ATGTAAAATT TTTAAAGACT ATATGGTGT 2100
 CTTACGGTTT TACATCTAGT AAGAGATTAA AAAAAAAAAA AAAAGCAAGG AAAGTAAGTG 2160
 TAAAGAGAGT AAAGAGAATG TAGCCATGAT ATGGCTGATT GTTCATCACC ATCCCATTTA 2220
 TACTTATCAT CTTGATGATG CATATAGACA AACACACTAC TTATACAGAT GTAGCATGTC 2280
 TCAGCTCCAA ATGGTGATCT TCTCCTGGCA TAACCTCTTA GATGTCACCT CCTCCTTGAT 2340
 CTTCTCCAC TATAAAACCA GCTAGTTCAC AACACCTATT CACCACATCA CATCCCATTC 2400
 C 2401

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1732 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GGATCCTGTA AGAAGTGCCC AAAATGTGAG AAGTGTATTA TAACACTATA TATAATACTA 60
 TATAACACCA TATAAATACC GTATAACACT ATGTAACACC ATATAACACA ATATAACGCT 120
 ATGTAACACT ATATAACATT ATATAACAAT ATATAACACT ATACATCTAT CAGAGACATG 180
 CTATCAGACA ACCTATAGTG TTATATTTGT TATATAATGT TATATAGTGT TACATAGCGT 240
 TATATGGTAT TATATGGTGT TACATATTGT TATACGTGTT TATATGGTGT TATATAGTAT 300
 TATATATAGT GTTATAATAC ACTTCTCACA CTTTGGGCAC TTTTACAGG ATCATCTACC 360
 TATATATATA TATATATATA TAAAGGATTA GGTTCAAACG TGAACAAATT CCCAAGAGTG 420
 AACTGCGTGA ACTGATCTCA GCCCTTGATT TTTATGATCT TGAGATTAAA GTGAGTGCGA 480

-43-

TGATGGTAAT TATTTGGTTA ATTTTTTTTC ATTTAATTAA ATACAAAAAG GGTATATGTG	540
TAATTTCAAT CTTAAATTGA TTGCATAAAT CTCTCACAAA TCAAGTAATC AATTATCTTC	600
TTAAACTGAT TACATAAATC TCTCACAAAT CAAATCAAGG ATTAGGAAAG ATGTAACCTA	660
ATTCTAATTA CTAAATAAC TATTTGTTTA AATGCGATGT ACACATGTGT ATTCTGATTT	720
TGCCCTCTTT TTAATGTGAT GTACACATGT GTATATCGTC TGTTTTTATG AGATCTCAGA	780
ATTTTTTTTG TATTGAATGT TGATGTACAC CTGTGAATTA CTGTACACAT ATGTACGATG	840
CTGATGCTGA GTACACATGT GTECTGTTCT ATTTATATCC AAGTACACAT GTGTAACCTT	900
GAAATATGAA AGTTACGTGG ATCTTAAAAA TCAAAATTTG AATTCTGGTG ATGAAATCTG	960
AAATAAAAT TAAAATTGAA ATCTGGTGAT TTGTTGTTTG TTTTGATAAT TATCTTATTA	1020
ATAAATAAC ATAATGTGGA TAATGAATTT AAATTAGGAA AGATGTAAC TAATTCAATT	1080
ATTAAAATAA TGATTTAAAT CTAATTTTTT ATATAATTAC AATCCTACCC TTAACAATA	1140
AAAAGGAAAT CAAGGGTTCA TATCTGTTCA CGCAGTTCAC TCTTGGGAGG TTGTTACGCG	1200
TGGAACCCTA CCCTATATAT ATATATATAT ATATATCAA TTTTTTTAAA AAATCCCGTA	1260
CCCCTCGAAA AAACGGGCCT TATGCGGAAG TCCTCCTCGC ACACCTAAAG AGCCGCCCAT	1320
GCTTTTGATC AAATAGTTGT AAATACTAAA ATACACCAAG TCAATGGGTG AAAGTTACTA	1380
TCTTTTTTAT TGCAATTTCA CATTACCTTA TTTACTTTTG AGAAAGACGA CATAACAATT	1440
AAGGAGTTAT AGTCTGATCG TTTGCGCTAT TTTTCATACT TAAGGTCCAG GTTTGAATAT	1500
TTTAAACATT TTTTTTAACT TGATCATAAC AATATAACAA TTAAGGAGTT ATGGTCTGAT	1560
GGTTTGC GTT ATGTTTTTCGT ACTAATTAAG GTCCCGGTTT GAATCTCTCA AACAATATAT	1620
TATTTTTACC TAAAAACGAA TGAGGCATGC TCACAAATGGG AATTGAACCG ACACCTATTG	1680
GTTTAAATTA AAAGCTATAA CAACTGAGC TACACATTTT TAATTTAAAA AT	1732

(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 404 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: both
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GTCGACTATC TTAGTTAATC AAATAAATTT ATTTTGATTT GTTTTGTTAA TGTATTTTCT 60

CCTAGTTTAA AGTCGATGTG TATTTATATA ATATTAGTAA TATTTTATTA ACATCAATAC	120
ATGCTTCAGG TTTTGTGTGA GTCTTCGTTT TTTATATGGT TTTATCAGCG GTSTGGTSTA	180
CGATGACGAT TATTTAAATA ATGACGGACT TCTTGGTTGT TACTTATTGA TGTACGAAGC	240
TGAGATGTAA CGAACCGAAC ACATATAAAT AACATTTTGG ATAAGATTAC GACTTTATT	300
ATCGGTTGCC ATGAAATTG GAAGACTTGG GTTAAGACAC AACCACATAT AATGTGATGG	360
TAAATAGCAT TTACAACATA TGTTAATCTT TTGTTACAAA TGTT	404

1 WHAT IS CLAIMED:

5 1. An isolated nucleic acid from a helianthinin gene comprising at least one regulatory element which directs at least one of seed-specific gene expression, root-specific gene expression, abscisic acid (ABA)-responsive gene expression and temporally-altered gene expression.

10 2. The nucleic acid of Claim 1 wherein said helianthinin gene is selected from an Ha2 gene, an Ha10 gene, an HaG3A gene or an HaG3D gene.

3. The nucleic acid of Claim 1 wherein said regulatory element is selected from an Ha2 gene, an Ha10 gene, an HaG3A gene or an HaG3D gene.

15 4. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs seed-specific gene expression is characterized in that expression of a gene under its control is detectable in seeds.

20 5. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs root-specific gene expression is characterized in that expression of a gene under its control is detectable in plant roots.

25 6. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs ABA-responsive gene expression is characterized in that expression of a gene under its control is detectable in response to treatment with ABA or conditions which induce ABA biosynthesis.

30 7. The nucleic acid of Claim 1, 2 or 3 wherein the regulatory element which directs temporally-altered gene expression is characterized in that expression of a gene under its control is detectable in plant seeds as early as 4 days post-flowering.

1 8. The nucleic acid of Claim 1 or 4 wherein said
regulatory element directs seed-specific gene expression and
comprises nucleotides 1 to 2401 or 851 to 2401 of SEQ ID NO:1.

5 9. The nucleic acid of Claim 1 or 5 wherein said
regulatory element directs root-specific gene expression and
comprises nucleotides 1 to 1639 or 851 to 1639 of SEQ ID NO:1.

10 10. The nucleic acid of Claim 1 or 6 wherein said
regulatory element directs ABA-responsive gene expression and
comprises nucleotides 1 to 2401 of SEQ ID NO:1, nucleotides 851
to 1639 of SEQ ID NO:1, nucleotides 1639 to 2303 of SEQ ID NO:1
or nucleotides 1 to 404 of SEQ ID NO:3.

15 11. The nucleic acid of Claim 1 or 7 wherein said
regulatory element directs temporally-altered gene expression
and comprises nucleotides 1 to 851 or 1639 to 2303 of SEQ ID
NO:1.

20 12. The regulatory of any one of Claims 1-11 wherein
said regulatory element is operably linked to the coding
sequence of a heterologous gene to effect said expression of a
gene product from said coding sequence and to provide a chimeric
plant gene.

25 13. The chimeric plant gene of Claim 12 comprising
a sufficient part of a promoter capable of functioning in plants
and operably linked to said coding sequence and said regulatory
element to effect expression of said heterologous gene.

30 14. The chimeric plant gene of Claim 25 wherein said
promoter is a plant virus promoter or the cauliflower mosaic
virus (CaMV) 35S promoter.

35 15. The chimeric plant gene of Claim 14 wherein said
promoter is the CaMV 35S promoter comprising CAAT and TATA
sequences.

 16. The chimeric plant gene of Claim 12 wherein said
heterologous gene is a gene encoding a lipid metabolism enzyme,
a desaturase, a herbicide resistance gene, a glyphosate

-47-

1 resistance gene or a gene encoding 5' enolpyruvylshikimic acid-
3-phosphate synthase, acetolactase synthase, or acetohydroxy
acid synthase.

5 17. The chimeric gene of Claim 16 wherein said
glyphosate resistance gene is aroA.

18. The chimeric gene of Claim 17 which comprises the
chimeric plant gene of pRPA-ML-803.

19. A plant transformation vector which comprises the
chimeric plant gene of any one of Claims 12-18.

10 20. A plant cell comprising the transformation vector
of Claim 19.

21. A plant, or a progeny of said plant, which has
been regenerated from the plant cell of Claim 20.

15 22. The plant of Claim 21 wherein said plant is a
cotton, tobacco, oil seed rape, maize or soybean plant.

23. The plant cell of Claim 20 wherein said plant
cell is a cotton, tobacco, oil seed rape, maize or soybean plant
cell.

20 24. A method for producing a plant with improved seed
lipid quality which comprises:

a) transforming a plant cell with the transformation
vector of Claim 19; and

b) regenerating said plant with improved seed lipid
quality from said transformed plant cell.

25 25. A method for producing a plant which exhibits
resistance to a herbicide which comprises:

a) transforming a plant cell with the transformation
vector of Claim 19; and

30 b) regenerating said plant from said transformed
plant cell.

26. The use of regulatory element of any one of
Claims 1-12 for producing transgenic plants.

1 27. The use of the nucleic acid of any one of Claims
1-11 for producing transgenic plants.

5

10

15

20

25

30

35

1 / 12

	-60		-50		-40	
-2377						GGATCCT
-2340	TTTTAAAAAA		TCCCGTACCC		CTCGAAAAAA	
-2280	CCTAAAGAGC		CGCCCATGCT		TTTTAATCAA	
-2220	ACTAAATCC		ATTAGTTTAT		AAATATTTTA	
-2160	AAATTA AAAA		ACAAATGTC		CAAAATACTC	
-2100	CCCTTTTGGT		TAACTAAGTC		TTTAATTTAA	
-2040	AAAAACGAAC		GTGGGGGCGC		GGGGGTGGGG	
-1980	TTAAAGTATA		AAATTTGCC		AAACCTCAGG	
-1920	AATACTAAAA		TACACCAAGT		CAATGGGTGA	
-1860	ATTACCTTAT		TTACTTTTGA		GAAAGACGAC	
-1800	TTTGCGCTAT		TTTTCATACT		TAAAGTCCAG	
-1740	TTGATCATAA		CAATATAACA		ATTAAGGAGT	
-1680	TACTAATTAA		GGTCCCGGTT		TGAATCTCTC	
-1620	ATGAGACATG		CTCACAATGG		GAATTGAACC	
-1560	ACAAACTGAG		CTACACATTT		TTAATTTAAA	
-1500	TTTATTTTGA		TTTGTTTTGT		TAAATGATTT	
-1440	ATAATATTAG		TAAATTTTTA		TTAACATCAA	
-1380	TTTTTTATAT		GGTTTTATCA		GTGGTGTGGT	
-1320	ACTTCTTGGT		TGTTACTTAT		TGATGTACGA	
-1260	AATAACATTT		TGGATAAGAT		TACGACTTTA	
-1200	TGGGTTAAGA		CACAACCACA		TATAATGTGA	
-1140	CTTTTGTTAC		AAATGTIGTT		AACTAGGCTT	
-1080	GTTCTTACGG		TTTTACATCT		AGTAAGAGAT	
-1020	AAAGAGAGTA		AAGAGAAATG		AGCCATGATA	
-960	ACTTATCATC		TTGATGATGC		ATATAGACAT	
-900	TTCCCGGCGC		AACACACGTG		TATAAATACC	
-840	GGTGGTTATA		TGATACTTAT		GATGACTTGA	
-780	AAAAAATGTG		GCTATAGGCG		CATGGTCACA	
-720	ACGTAGCGTT		AGTTAATCAG		ATAAATTTAT	
-660	TAAATTC AAG		TAGACGTGTA		TTTATATAAT	
-600	GCTTCATGTT		TTGGGTTAGT		CTTCGTTTTT	
-540	GATTATTTAA		ATAATGACGG		ACTTCTTGGT	
-480	TAAACGAACCG		AACACATATA		AATAACATTT	
-420	GCCATGAAAT		TTGGAAGACT		TGGGTTAAGA	
-360	CATTTACAAC		TAAATGTTAA		CTTTTGTTAC	
-300	ATTTTTAAAG		ACTATATGGT		GTTCTTACGG	
-240	AAAAAAGCA		AGGAAAGTAA		GTGTAAAGAG	
-180	ATTGTTTATC		ACCATCCCAT		TTATACTTAT	
-120	TACTTATACA		GATGTAGCAT		GTCTCAGCTC	
-60	TTAGATGTCA		CTTCCTCCTT		GATCTTCTTC	
1	ATTCACCACA		TCACATCCCA		TTCC	
		10		20		30

FIG. 1

SUBSTITUTE SHEET

2 / 12

-30 | -20 | -10 |
CTACCTATAT ATATATATAT ATATGAATTT -2341
CGGGCCTTAT GCGGAAGTCC TCCTCGCACA -2281
ATAGATGTGC ATCATGTAGT GATAGTTTTT -2221
AATGTTTTTT TTTGTTTATA TAAAAAAGA -2161
CTGTATCAAC TATGCALAAA GACAAAAAAA -2101
CTAAGTTTGT CATTGGAAGG AATTCALAC -2041
TGTTTGGTTA CAAAAAGTTT TAATTTTAGA -1981
ACAATTTTTA CATTATATAC TCATTGTCTA -1921
AAGTTACTAT CTTTTTTATT GCAATTTTAC -1861
ATAACAATTA AGGAGTTATA GTCTGATCGG -1801
GTTTGAATCT TTTAAACATT TTTTTTAAAC -1741
TATGATCTGA TGGTTTGGCT TATGTTTTCG -1681
AAACAATATA TTATTTTTTC TAAAAAACGA -1621
GACACCTATT GGTTTAAAAAT TAAAGCTATA -1561
AATGTCGACT ATCTTAGTTA ATCAAAATAA -1501
TCTCCTAATT TAAAGTCGAT GTGTATTTAT -1441
TACATGCTTC AGGTTTTGTG TTAGTCTTCG -1381
GTACGATGAC GATTATTTAA ATAATGACGA -1321
AGCTGAGATG TAACGAACCG AACACATATA -1261
TTTATCGGTT GCCATGAAAT TTAGAAGATT -1201
TGGTAAATAG CATTACAAAC TAATGTTAAT -1141
GATATGTAAA ATTTTAAAG ACTATCAGGT -1081
TAAAAAANA AAAGCAAGGA AAGTAAGTGT -1021
TGGCTGATTG TTCATCACCA TCCCATTAT -961
GATGTGTGCT ACGTACCGAA TTTTAAACAGC -901
ATAGATTATA AACCAATAC GCTACGTATA -841
CCTTTCGTTA CACTTGAGCT GAAAAAATA -781
GTTTTTTTGT GTGGCCATAT ACAATTTTTG -721
TTTGATTTGT TTTGTTAATG TATTTTCTCC -661
ATTAGTAATA TTTATTAAC ATCAATACAT -601
TATATGGTTT TATCAGTGGT GTACGATGAC -541
TGTTACTTAT TGATGTACGA AGCTGAGATG -481
TGGATAAGAT TACGACTTTA TTTATCGGTT -421
CACAAACCACA TATAATGTGA TGGTAAATAG -361
AAATGTTTGT AACTAGGCTT GATATGTAAA -301
TTTTACATCT AGTAAGAGAT TAAAAAANA -241
AGTAAAGAGA ATGTAGCCAT GATATGGCTG -181
CATCTTGATG ATGCATATAG ACAACACAC -121
CAAATGGTGA TCTTCTCCTG GCATAACCTC -61
CACTATAAAA CCAGCTAGTT CACAACACCT -1
24
| 40 | 50 | 60

FIG. 1 CONT.'

SUBSTITUTE SHEET

3 / 12

```
      -60      |      -50      |      -40      |  
-2457      GGATCCT  GTAAGAAGTG  CCCAAAAATGT  
-2400      CTATATAACA  CCATATAAAT  ACCGTATAAC  
-2340      GCTATGTAAC  ACTATATAAC  ATTATATAAC  
-2280      ATGCTATCAG  ACAACCTATA  GTGTTATATT  
-2220      CGTTATATGG  TATTATATGG  TGTACATAT  
-2160      TATTATATAT  AGTGTTATAA  TACACTTCTC  
-2100      ACCTATATAT  ATATATATAT  ATATAAAGGA  
-2040      GTGAACTGCG  TGAACCTGATC  TCAGCCCTTG  
-1980      GCATGATGGT  AATTATTGGG  TTAATTTTTT  
-1920      GTGTAATTTT  AATCTTAAAT  TGATTGCATA  
-1860      TTCTTAAACT  GATTACATAA  ATCTCTCACA  
-1800      TTAATTCTAA  TTAATAAAT  AACTATTTGT  
-1740      TTTTGCCCTC  TTTTAAATGT  GATGTACACA  
-1680      AGAATTTTTT  TTGTATTGAA  TGTGTATGTA  
-1620      ATGCTGATGC  TGAGTACACA  TGTGTACTGT  
-1560      CTTGAAATAT  GAAAGTTACG  TGGATCTTAA  
-1500      CTGAAATAAA  AATTAALATT  GAAATCTGGT  
-1440      TTAATAAATA  AACATAATGT  GGATAATGAA  
-1380      ATTATTAAAA  TAATGATTTA  AATCTAATTT  
-1320      CTAAMMAGGA  AATCAAGGGT  TCATATCTGT  
-1260      CGCTGGAACC  CTACCCTATA  TATATATATA  
-1200      GTACCCCTCG  AAAAAACGGG  CCTTATGCGG  
-1140      CATGCTTTTG  ATCAATAAGT  TGTAAATACT  
-1080      CTATCTTTTT  TATTGCAATT  TCACATTACC  
-1020      ATTAAGGAGT  TATAGTCTGA  TCGTTTGGCG  
-960      TATTTTAAAC  ATTTTTTTTA  ACTTGATCAT  
-900      GATGGTTTGC  GTTATGTTTT  CGTACTAATT  
-840      TATTATTTTT  ACCTAAAAAC  GAATGAGGCA  
-780      TTGGTTTAAA  ATTAAGCTA  TAACAACCTG
```

FIG. 2

SUBSTITUTE SHEET

4 / 12

```
-30      |   -20      |   -10      |  
GAGAAGTGTA TTATAACACT ATATATAATA -2401  
ACTATGTAAC ACCATATAAC ACAATATAAC -2341  
AATATATAAC ACTATACATC TATCAGAGAC -2281  
TGTTATATAA TGTTATATAG TGTTACATAG -2221  
TGTTATACGT GTTTATATGG TGTTATATAG -2161  
ACACTTTGGG CACTTTTTAC AGGATCATCT -2101  
TTAGGTTCAA ACGTGAACAA ATTCCCAAGA -2041  
ATTTTTATGA TCTTGAGATT AAAGTGAGTG -1981  
TTCATTTAAT TAAATACAAA AAGGGTATAT -1921  
AATCTCTCAC AAATCAAGTA ATCAATTATC -1861  
AATCAAATCA AGGATTAGGA AAGATGTAAC -1801  
TTAAATGCGA TGTACACATG TGTATTCTGA -1741  
TGTGTATATC GTCTGTTTTT ATGAGATCTC -1681  
CACCTGTGAA TTA CTGTACA CATATGTACG -1621  
TCTATTTATA TCCAGTACA CATGTGTAAC -1561  
AAATCAAAAT TTGAATTCTG GTGATGAAAT -1501  
GATTTGTTGT TTGTTTGTAT AATTATCTTA -1441  
TTTAAATTAG GAAAGATGTA ACTTAAATCA -1381  
TTTATATAAT TACAATCCTA CCCTTAAACA -1321  
TCACGCAGTT CACTCTTGGG AGGTTGTTCA -1261  
TATATATATC AAATTTTTTT AAAAAATCCC -1201  
AAGTCCTCCT CGCACACCTA AAGAGCCGCC -1141  
AAAAACACC AAGTCAATGG GTGAAAGTTA -1081  
TTATTTACTT TTGAGAAAGA CGACATAACA -1021  
TATTTTTCAT ACTTAAAGTC CAGGTTTGAA -961  
AACAAATATA CAATTAAGGA GTTATGGTCT -901  
AAGGTCCCGG TTTGAATCTC TCAACAATA -841  
TGCTCACAAI GGAATTGAA CCGACACCTA -781  
AGCTACACAT TTTAATTTA AAAAT
```

FIG. 2 CONT.'

SUBSTITUTE SHEET

5 / 12

-720 CTATCTTAGT TAATCAATA AATTATTTT
-660 TTTAAAGTCG ATGTGTATTT ATATAATATT
-600 TCAGGTTTTG TGTTAGTCTT CGTTTTTTAT
-540 ACGATTATTT AAATAATGAC GGACTTCTTG
-480 TGTAAACGAAC CGAACACATA TAAATAACAT
-420 TTGCCATGAA ATTTGGAAGA CTTGGGTTAA
-360 AGCATTITACA ACTAATGTTA ATCTTTTGTT

FIG. 3

SUBSTITUTE SHEET

6 / 12

GTCGA -721
GATTTGTTTT GTTAATGTAT TTTCTCCTAG -661
AGTAATATTT TATTACATC AATACATGCT -601
ATGGTTTTAT CAGCGGTGTG GTGTACGATG -541
GTTGTTACTT ATTGATGTAC GAAGCTGAGA -481
TTTGGATAAG ATTACGACTT TATTTATCGG -421
GACACACCA CATATAATGT GATGGTAAAT -361
ACAAATGTT

FIG. 3 CONT.'

SUBSTITUTE SHEET

7 / 1 2

FIG. 4

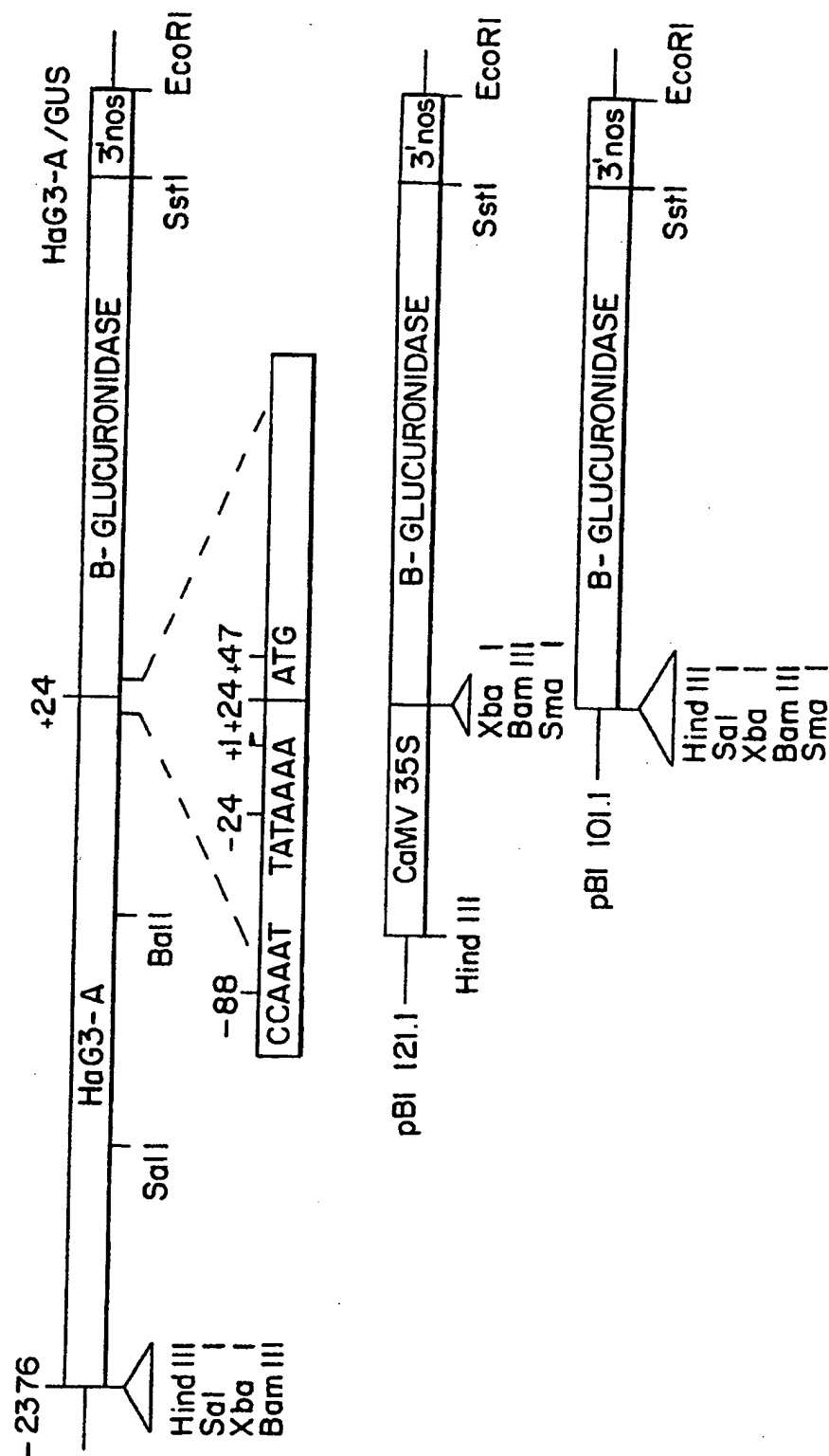
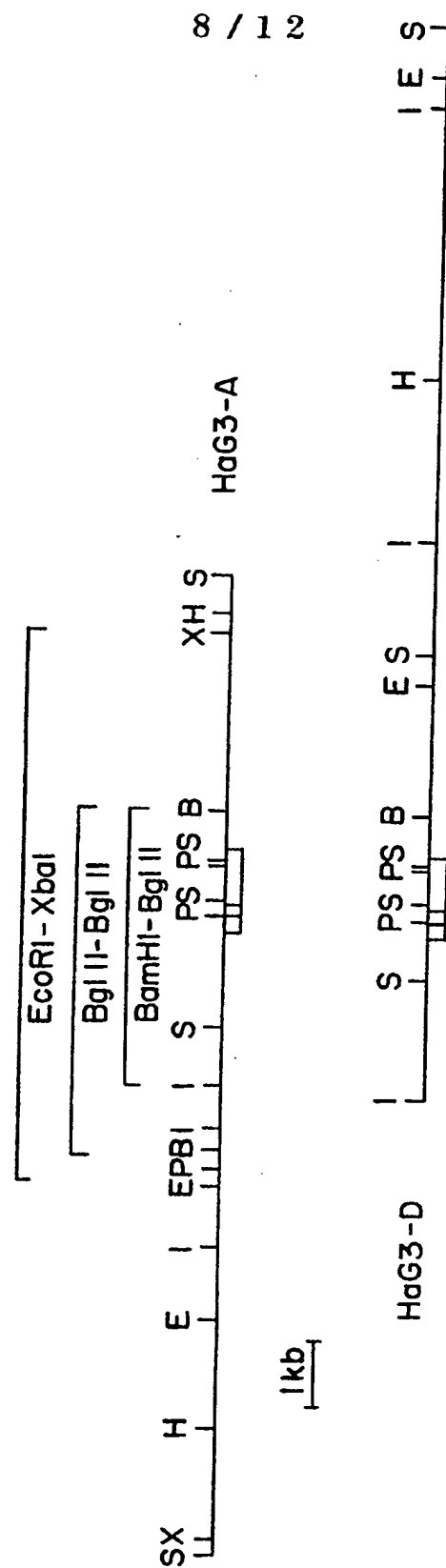
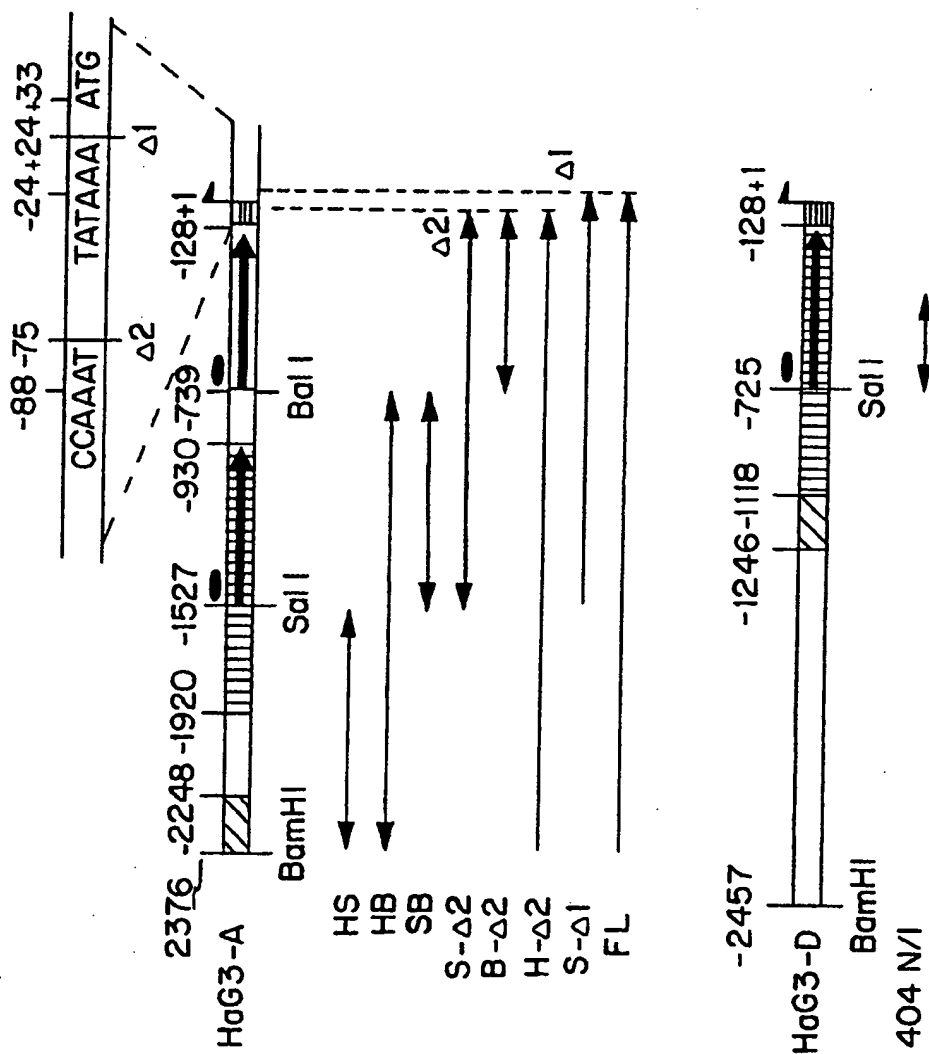


FIG. 5



9 / 12

FIG. 6



SUBSTITUTE SHEET

10 / 12



FIG. 7A

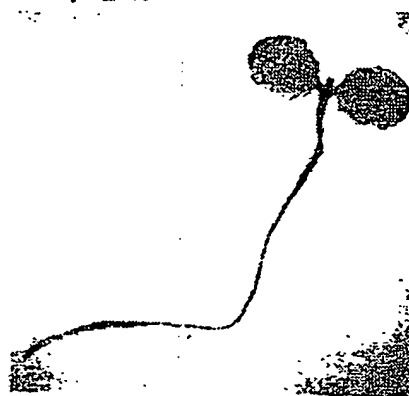


FIG. 7B

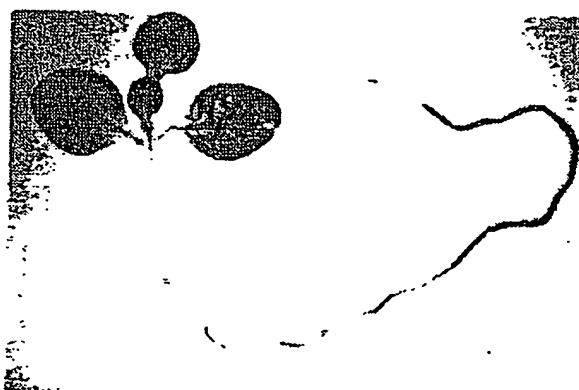


FIG. 7C



FIG. 7D

SUBSTITUTE SHEET

BEST AVAILABLE COPY

10/12/1

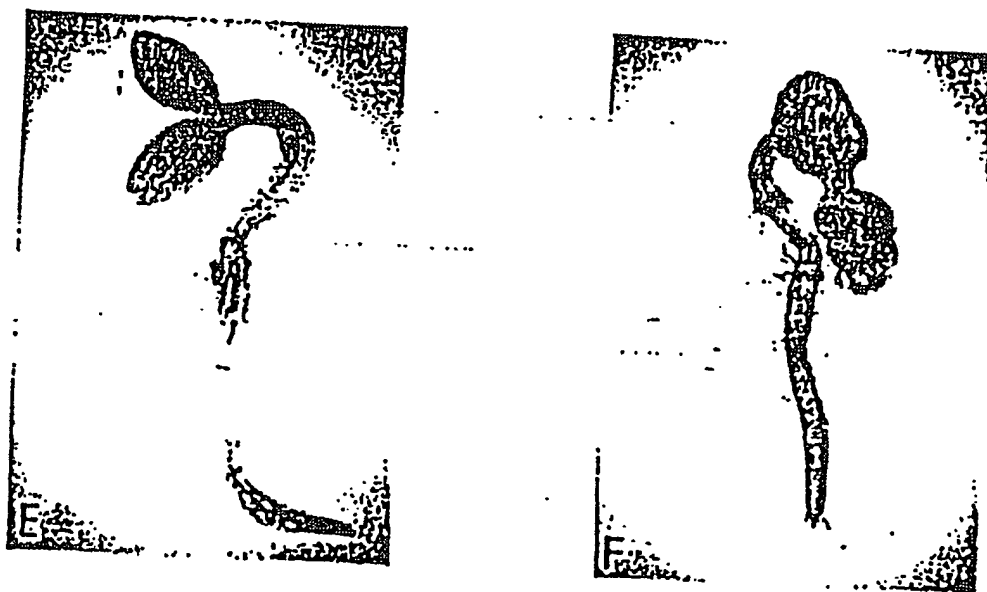


Figure 7

SUBSTITUTE SHEET

BEST AVAILABLE COPY _____

11 / 12

FIG. 8A

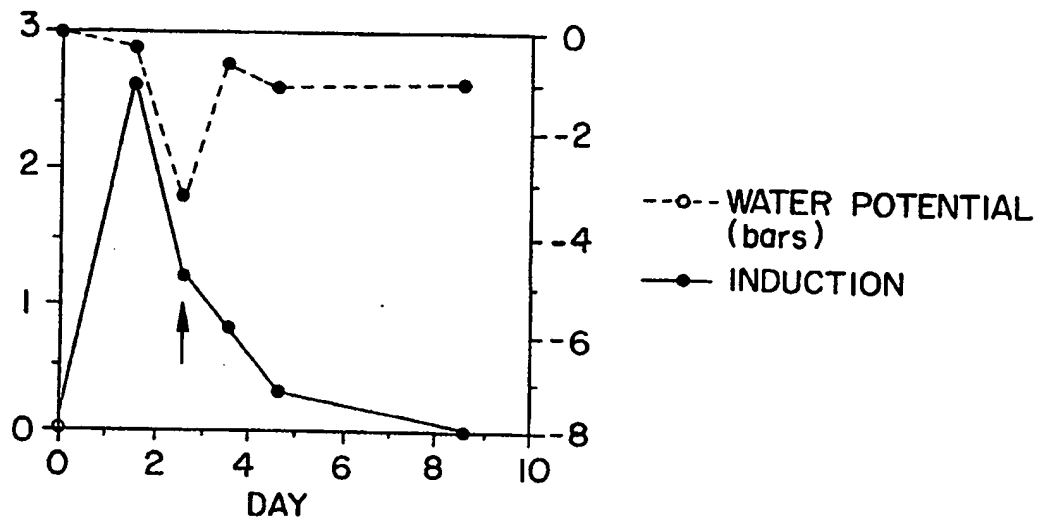
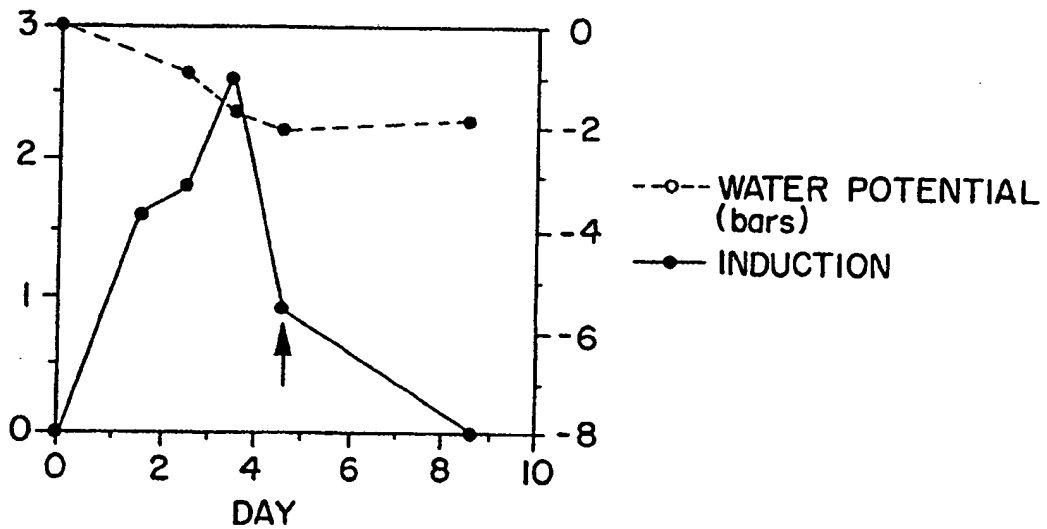


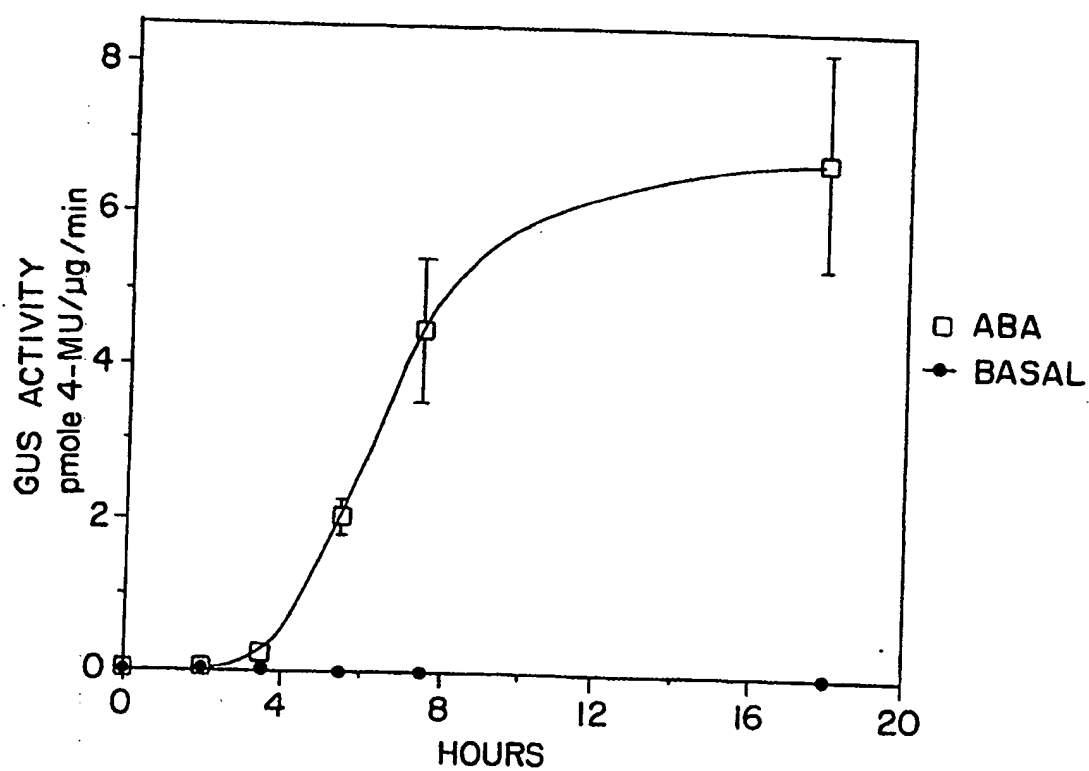
FIG. 8B



SUBSTITUTE SHEET

12 / 12

FIG. 9



SUBSTITUTE SHEET

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US92/02822

I. CLASSIFICATION OF SUBJECT MATTER (If several classification symbols apply, indicate all) ³ According to International Patent Classification (IPC) or to both National Classification and IPC IPC (5): C12N 05/10, 15/63, 15/82; C07H 15/12; A01H 5/00 US CL : 435/172.3, 240.4, 320.1; 536/27; 800/205		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁴		
Classification System	Classification Symbols	
U.S.	435/172.3, 240.4, 320.1; 536/27; 800/205, DIG. 69; 935/30, 35, 64, 67	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched ⁵		
APS, DIALOG search terms: helianthinin		
III. DOCUMENTS CONSIDERED TO BE RELEVANT ¹⁴		
Category ⁶	Citation of Document, ¹⁰ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
X/Y	The Plant Cell, Volume 1, issued September 1989, Jordano et al, "A sunflower helianthinin gene upstream sequence ensemble contains an enhancer and sites of nuclear protein interaction", pages 855-866, see entire document.	1-4, 8, 12-15, 19-23/16, 24
X/Y	Gene, Volume 74, issued 1988, Vonder Haar et al, "Organization of the sunflower 11S storage protein gene family", pages 433-443, see entire document.	1-4, 8 / 12-16, 19-24
Y	Trends in Biotechnology, Volume 5, issued February 1987, Knauf, "The application of genetic engineering to oilseed crops", pages 40-47, see entire document.	12-13, 16, 19-24
Y	EP. A. 0,255,377 (Kridl et al) 03 February 1988, see entire document.	12-13, 16, 19-24
Y	Trends in Genetics, Volume 4, No. 1, issued January 1988, Willmitzer, "The use of transgenic plants to study plant gene expression", pages 13-18, see entire document.	14-15
* Special categories of cited documents: ¹⁶ "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "Z" document member of the same patent family		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search ² 23 June 1992		Date of Mailing of this International Search Report ² 07 JUL 1992
International Searching Authority ¹ ISA/US		Signature of Authorized Official ⁷ P. R. RHODES

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

- | | | |
|---|---|----|
| Y | The Journal of Biological Chemistry, Volume 265, No. 33, issued 25 November 1990, Stukay et al, "The OLE1 gene of <i>Saccharomyces cerevisiae</i> encodes the Δ9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene", pages 20144-20149, see entire document. | 16 |
| Y | The Journal of Biological Chemistry, Volume 257, No. 20, issued 25 October 1982, McKeon et al, "Purification and characterization of the stearoyl-acyl carrier protein desaturase and the acyl-acyl carrier protein thioesterase from maturing seeds of safflower", pages 12141-12147, see entire document. | 16 |

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE¹

1. ☐ Claim numbers , because they relate to subject matter (1) not required to be searched by this Authority, namely:
2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out (1), specifically:
3. ☐ Claim numbers , because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING²

This International Searching Authority found multiple inventions in this international application as follows:
SEE ATTACHMENT

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims: 1-4, 8, 12-16, AND 19-24
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Search Authority did not invite payment of any additional fee.

Remark on protest

- ☐ The additional search fees were accompanied by applicant's protest.
☒ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category*	Citation of Document, ¹⁶ with indication, where appropriate, of the relevant passages ¹⁷	Relevant to Claim No. ¹⁸
Y	US, A, 4,394,443 (Weissman et al) 19 July 1983, see entire document.	16

The claims present a plurality of mutually exclusive independent inventions as follows:

I. Claims 1-3, 12-16, and 19-24, drawn to a DNA product and the vector, plant cell, and plant containing same and a first method of use, classified in Classes 435, 536, and 800; Subclasses 172.3, 240.4, and 320.1, 27, and 205, respectively, for example.

Note that the following are independent and distinct species pertinent to the invention of Group I where a) is the first species of the first component and e) is the first species of the second component and both of which will be searched with claims 1-3, 12-16, and 19-24 in the event that no other fees are paid. Note that a search of any other additional species within Group I requires payment of additional fees.

The first component:

- a) seed-specific regulatory element (claims 4 and 8);
- b) root-specific regulatory element (claims 5 and 9);
- c) ABA-responsive regulatory element (claims 6 and 10);
- d) temporally-altered regulatory element (claims 7 and 11);

The second component:

- e) the heterologous gene encodes a lipid metabolism enzyme;
- f) the heterologous gene encodes a desaturase;
- g) the heterologous gene encodes a herbicide resistance gene;
- h) the heterologous gene encodes *aroA* for glyphosate resistance (claims 17-18);
- i) the heterologous gene encodes EPSPS;
- j) the heterologous gene encodes acetolactase synthase;
- k) the heterologous gene encodes acetohydroxy acid synthase.

II. Claim 25, drawn to a second process of use, classified in Class 435, Subclass 172.3, for example.

III. Claim 26, drawn to a third process of use, classified in Class 435, Subclass 172.3, for example.

IV. Claim 27, drawn to a fourth process of use, classified in Class 435, Subclass 172.3, for example.

The inventions are distinct, each from the other because of the following reasons:

The claims of each of Groups I-IV for a method of use are distinct and independent as the search for each group is not coextensive for the different properties of the diverse products produced by these methods require different considerations for regulating expression and vector construction. Furthermore, the third method of use requires steps to identify regulatory subset sequences as well as a search of separate subject matter since the third and fourth methods of use are unspecified and since there are numerous methods of making transgenic plants and since considerations for construction and operability differ with regulatory element sequences as opposed to whole fragments containing various regulatory elements including silencers, for example. A search for any one group would not result in a complete and thorough search for any one other group.

PCT/ISA/210

PCT/US92/02822

Because these inventions are distinct for the reasons given above and have acquired a separate status in the art as shown by their recognized divergent subject matter and separate search requirements, lack of unity as indicated is proper.

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

☐ BLACK BORDERS

☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES

☐ FADED TEXT OR DRAWING

☐ BLURRED OR ILLEGIBLE TEXT OR DRAWING

☐ SKEWED/SLANTED IMAGES

☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS

☐ GRAY SCALE DOCUMENTS

☒ LINES OR MARKS ON ORIGINAL DOCUMENT

☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY

☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.